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CD8$^+$ T cell epitopes derived from the polyomavirus capsid protein VP1

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Dedicated to my Family!

TABLE OF CONTENTS

LIST OF ABBREVIATIONS v

LIST OF FIGURES vii

LIST OF TABLES ix

SUMMARY x

AIM OF THE STUDY xii

1 INTRODUCTION 1

1.1 Role of cellular immunity in viral infections 1

1.2 Antigen processing and presentation 2

1.2.1 MHC/HLA class I antigen presentation 3

1.2.2 Processing of VP1 protein, VLP 6

1.3 Immunosuppression and viral infections 7

1.4 JC and BK polyomaviruses 8

1.4.1 Morphology and genome 8

1.4.2 Seroprevalence and infection 9

1.4.3 JCV-associated complications and diagnosis 11

1.4.4 Adaptive immune responses to the JCV 13

1.4.4.1 Humoral immune response 13

1.4.4.2 Cellular immune response 14

1.4.5 Treatment of polyomavirus disease 15

1.5 Adoptive immunotherapy 16

1.6 Characterization of novel T cell epitopes (TCEs) 21

1.6.1 Strategies for T cell mapping 21
1.6.1.1 Overlapping peptides 21
1.6.1.2 Peptide matrices 21
1.6.1.3 *In silico* approach 22
1.6.2 Methods used for T cell epitope mapping: 22
2 MATERIAL AND METHODS 25
2.1 MATERIAL 25
2.1.1 Plasticware 25
2.1.2 Medium, Solutions and buffers 26
2.1.3 Solutions, buffers and antibodies for ELISPOT 26
2.1.4 Antibodies and tetramers for flow cytometry 27
2.1.5 Reagents and chemicals 27
2.1.6 Equipments 28
2.1.7 Primers 29
2.1.8 Peptides, peptide pool and proteins 29
2.1.9 Healthy donors (HDs) 30
2.1.10 Patient sample 31
2.1.11 Cell lines 31
2.2 METHODS 32
2.2.1 Human leukocyte antigen (HLA)-typing of HDs 32
2.2.1.1 Typing by antibodies 32
2.2.1.2 Typing by single specific primer-PCR (SSP-PCR) 32

2.2.2 Lympho-proliferative assay 33

2.2.2.1 Mixed lymphocyte peptide culture (MLPC) 33

2.2.2.2 “Whole PBMC” approach 35

2.2.3 CFSE proliferation assay 35

2.2.4 JCV-specific T cell screening 35

2.2.5 ELISPOT assay 36

2.2.5.1 IFN-γ secretion assay 36

2.2.5.2 Granzyme B secretion assay 37

2.2.6 Flow cytometry 38

2.2.6.1 Surface marker staining 38

2.2.6.2 IFN-γ intracellular staining 38

3 RESULTS 40

3.1 Characterization of the relative frequency of polyomavirus-specific T cells in the peripheral blood 40

3.2 Strategies 42

3.2.1 MLPC versus “whole PBMCs” approach 42

3.2.2 Optimization of T cell stimulation 44

3.2.2.1 Antigen presenting cells (APCs) 44

3.2.2.2 Peptide length and concentration 48

3.2.2.3 Peptide matrix sub-pools versus single peptide stimulation 51

3.3 Characterization of high-affinity JCV-p100-peptide-specific $CD8^+$ T cells .. 54

3.4 Cross-reactivity of $CD8^+$ T cells specific to JCV and BKV 58

3.5 HLA-typing of HDs 60

3.6 Pre-screening of JCV-VP1-specific T cells in HDs 62

3.7 Processing of JCV-VP1-VLP 64

3.8 Assessment of novel JCV-VP1 $CD8^+$ T cell epitopes (TCEs) 66

3.8.1 Novel T cell epitopes (TCEs) in HDs 66

3.8.2 Novel T cell epitopes (TCEs) in a patient 73

4 DISCUSSION 77

LITERATURE 90

ACKNOWLEDGEMENT 101

SUPPLEMENTARY 103

LIST OF ABBREVIATIONS

AEC	3-Amino-9-ethylcarbazole
ALP	Alkaline phosphatase
APC	Allophycocyanin
APCs	Antigen presenting cells
BBB	Blood brain barrier
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BKV	BK virus (polyomavirus)
BSA	Bovine serum albumin
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
CMV	Cytomegalovirus
CTLs	Cytotoxic T lymphocytes
DLIs	Donor lymphocyte infusions
DMSO	Dimethyl sulfoxide
dNTPs	Deoxynucleotide triphosphates
EBV	Epstein-Barr virus
ELISPOT	Enzyme-linked immunospot
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GM1	Ganglioside-monosialic acid
GMP	Good manufacturing practice
HAART	Highly active antiretroviral therapy
HC	Hemorrhagic cystitis
HDs	Healthy donors
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
HSCT	Hematopoietic stem cell transplantation
HSV	Herpes simplex virus
HTS	High throughput screening

ICS	Intracellular cytokine staining
IFN-γ	Interferon-gamma
IKTZ	Institut für Klinische Transfusionsmedizin und Zelltherapie
IL	Interleukin
JCV	JC virus (polyomavirus)
LT-antigen	Large T-antigen
MACS	Magnetic-activated cell sorting
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MLPC	Mixed lymphocyte peptide culture
MS	Multiple sclerosis
NBT	Nitro blue tetrazolium chloride
OP	Overlapping peptide
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PDI	Protein disulphide isomerase
PE	Phycoerythrin
PHA	Phytohemagglutinin
PML	Progressive multifocal leukoencephalopathy
rpm	Revolutions per minute
RPMI-1640	Roswell Park Memorial Institute
RT	Room temperature
SEB	Staphylococcal enterotoxin B
SLE	Systemic lupus erythematosus
ST-antigen	Small T-antigen
SV-40	Simian virus-40
TAP	Transporter associated with antigen processing
TCR	T-cell-receptor
TNF-α	Tumor necrosis factor- α
UV	Ultra violet
VLP	Virus like particles

LIST OF FIGURES

Figure 1: Sources of antigen for MHC class I and class II presentation. 3

Figure 2: MHC/HLA class I processing and presentation. 5

Figure 3: Organization of the genome of human polyomaviruses. 9

Figure 4: Clinical course of human polyomavirus infection..... 10

Figure 5: Overview of antigen-specific immunotherapeutic strategies. 17

Figure 6: Scheme representing MLPC procedure. 34

Figure 7: General scheme representing ELISPOT assay..... 37

Figure 8: Comparison of MLPC versus “whole PBMCs stimulation”..... 43

Figure 9: Comparison of APCs..... 45

Figure 10: Antigen presentation properties of K562 wild type (non-transfected) and transfected cells 47

Figure 11: Comparison of 9mer versus 15mer induced T cell response.. 49

Figure 12: 9mer versus 15mer peptide, the concentration matters. 50

Figure 13: Overlapping peptide sequences and matrix peptide sub-pools covering the JCV-VP1 sequence. 52

Figure 14: Comparison of T cell stimulation by single peptides versus matrix sub-pools..... 53

Figure 15: Detection of high-affinity T cells. 55

Figure 16: Long-term culture of JCV-VP1-p100-peptide-specific $CD8^+$ T cells. 57

Figure 17: Alignment of JCV-VP1 versus BKV-VP1 using the data base protein BLAST (blast.ncbi.nlm.nih.gov). 58

Figure 18: TCR cross-reactivity between T cells specific for JCV and BKV peptides. 59

Figure 19: HLA-typing by flow cytometry. 61

Figure 20: Gel electrophoresis of SSP-PCR products. 61

Figure 21: Pre-screening of HDs, based on proliferation and IFN-γ secretion 63

Figure 22: Analysis of JCV-VLP processing. 64

Figure 23: Analysis of VLP processing using the OP25 peptide. 65

Figure 24: Systematic scheme of T cell epitope mapping 67

Figure 25: T cell epitope screening using matrix peptide sub-pools. 68

Figure 26: T cell responses to JCV and BKV in a HLA-B7 positive patient 74

Figure 27: TCE mapping in a patient. 76

LIST OF TABLES

Table 1: Studies reporting BKV and JCV-specific HLA-A2 restricted peptide sequences.. 20

Table 2: Relative frequency of JCV, CMV and influenza virus-specific $CD8^+$ T cells. 41

Table 3: Antigenic peptides tested in HLA-A2 positive healthy donors (HDs). 70

Table 4: Antigenic peptides tested in ten HLA-B7 positive healthy donors (HDs). ... 71

Table 5: Donor frequencies specific to potential TCEs..... 72

Table 6: HLA-typing of patient blood sample..... 73

SUMMARY

Reactivation of polyomaviruses JCV and BKV in immunocompromised patients after transplantation results in severe complications related to the central nervous system (CNS) and the urogenital system, i.e. progressive multifocal leukoencephalopathy (PML) and hemorrhagic cystitis (HC). Anti-viral drug treatment in this situation is not effective and therefore cure is largely dependent on the restoration of the immune system by tapering out of immunosuppressive drugs. However, restoration of immune system post transplantation might lead to graft versus host disease (GvHD). Adoptive transfer of virus-specific T cells constitute an alternative would be which would avoid the risk of GvHD due to its specific reactivity to virus-infected cells.

To this end, two previously known immunodominant human leukocyte antigen (HLA)-A2 restricted epitopes (p36 and p100) derived from VP1 protein were analyzed. We observed that the precursor T cell frequency to these two HLA-A2 restricted peptides was very low when compared to T cells specific for other viruses (e.g. CMV, influenza virus etc.). To efficiently measure polyomavirus-specific T cells *in vitro*, optimization towards increased sensitivity of the expansion protocol and the read-out system was performed. Further characterization showed that p100-specific T cells were T cells of high avidity. Moreover, in the *in vitro* culture, virus-specific T cells were successfully maintained for a longer period of time (up to 5 weeks) with no loss of peptide-specificity and functional reactivity. Additionally, we demonstrated that JCV-VP1-peptide-specific T cells were cross-reactive to the BKV-VP1-p108-peptide when measured by multi-parametric flow cytometry and functional assays like ELISPOT and cytotoxicity assays.

In a comprehensive approach to widen our knowledge for new $CD8^+$ TCEs restricted to other HLA-types, we used an array of overlapping peptides spanning the whole JCV-VP1 protein. Peptides were used in a form of matrix sub-pools and/or as individual peptide stimulation. In addition to two known HLA-A2 restricted peptide epitopes, several novel T cell specificities were identified in healthy donors (HDs) and in a patient. Using VP1-derived virus like particles (VLP) for initial stimulation, it was confirmed that all responding peptides were naturally processed. Additionally, a restriction analysis was performed for HLA-A2 and HLA-B7 alleles using HLA class I transfected lines of K562 and T2 cells as target cells in the immunological assays.

Taken together, we observed JCV-VP1-specific T cells to two HLA-A2 restricted immunodominant peptides of high avidity with sustained peptide-specificity and high functional reactivity in long-term cultures. Furthermore, we identified novel, naturally processed JCV-VP1-derived $CD8^+$ T cell epitopes (TCEs) restricted to HLA-A2 and HLA-B7. This new set of data will broaden the armamentarium of therapeutically useful peptides and contribute to the development of T cell immunotherapies against polyomavirus-related disease. Our long term goal includes enrichment and isolation of polyomavirus-specific T cell specificities for use as adoptive T cell transfer in the clinical setting. Cross-reactive T cells have the additional benefit that an appropriate immunotherapy can target both polyomaviruses simultaneously.

AIM OF THE STUDY

For JCV and BKV-associated diseases in immunocompromised patients, adoptive T cell transfer constitutes an excellent option. To date two immunodominant $CD8^+$ T cell epitopes have been identified in HLA-A2 individuals. To provide adoptive T cell transfer to patients across all haplotypes, there is a fervent need to identify and characterize more T cell epitopes. In the clinical setting, this extended knowledge would open new avenues for the efficient development of a comprehensive T cell immunotherapy against JCV and BKV infection.

The specific aim of this study was as follows:

1) Characterization of immunodominant HLA-A2 restricted JCV-VP1-p100-peptide-specific T cells.
2) Characterization of the cross-reactivity between JCV and BKV-peptide-specific T cells.
3) Identification of novel, naturally processed HLA-A2 and HLA-B7 restricted $CD8^+$ T cell epitopes in healthy donors (HDs) and patients.

1 INTRODUCTION

1.1 Role of cellular immunity in viral infections

Anti-viral cellular immunity differs from that of humoral immunity which is effective through antibodies. These soluble antibodies bind to the complex viral particles and can neutralize the infectivity of the virus. Also, antibodies cause viral elimination with the help of complement proteins. However, cell-mediated immunity is the most relevant mechanism in the control of viral infection or reactivation. With the help of T cell receptors (TCRs), T cells recognize virus-infected or-ingested cells and destroy these cells. Both $CD4^+$ and $CD8^+$ T cells actively participate in antiviral response. In immunocompetent individuals, cellular immunity keeps check on reactivation of latent viruses like cytomegalovirus (CMV), Epstein-Barr virus (EBV), adenoviruses, polyomaviruses etc.

The primary induction of cellular immune responses occurs in secondary lymphoid organs where antigen presenting cells (APCs) display antigens that are derived from viruses to T cells. APCs take up the viral antigens from the initial sites of infection. In secondary lymphoid organs, the encounter of naïve T cells with APCs leads to the activation of T cells. This results in the initiation of molecular and cellular signals including the release of cytokines and inflammatory mediators which are also important for the activation of other immune cells. T cells recognize virus-derived peptides or proteins only when these are associated with major histocompatibility complex (MHC) molecules, in humans also called human leukocyte antigen (HLA) molecules. Therefore, induced immune response is highly specific and the

magnitude depends greatly on the strength and duration of antigenic stimulation, HLA restriction, co-stimulatory signals and help of $CD4^+$ T cells.

$CD8^+$ T cells are the most important effectors cells of cellular immunity. These cells can undergo massive proliferation following virus encounter. $CD8^+$ T cells are activated in secondary lymphoid organs and later dispersed throughout the host body. Here, they produce inflammatory mediators like interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) and cytotoxic molecules (perforin, granzyme B) which kills the virus-infected cells before the release of progeny viruses.

$CD4^+$ T cells are helper T cells because they can help B cells and $CD8^+$ T cells for the successful activation of immune response. Additionally, $CD4^+$ T cells produce chemokines and cytokines that attract and stimulate other immune cells to the sites of infection. Furthermore, $CD4^+$ T cells can act as cytotoxic T cells like $CD8^+$ T cells which can directly lyse virus-infected cells.

The most astonishing feature of T cells is their potential to establish a memory T cell pool of virus-experienced cells. These memory T cells, which belong to less than 10% of the surviving T cells, are long-lived cells which are responsible for the rapid response generated during the subsequent attack of same virus. In an ideal situation, the memory T cells respond quickly and clear the infection much faster than the initial infection.

1.2 Antigen processing and presentation

Adaptive immune response starts off with the activation of effector B and T lymphocytes by APCs. Both forms of effector lymphocytes identify foreign antigens

with the help of highly variable antigen recognition receptors. An antigen to be recognized by T lymphocytes needs to be presented along with a specialized glycoprotein molecule called HLA class I or class II. Under normal circumstances, the origin of the antigen decides to which HLA molecule the peptide will bind and eventually which type of T cells ($CD4^+$ or $CD8^+$) will be activated. HLA class I molecules binds to the endogenous antigens found in the cytosol and stimulates $CD8^+$ T cells, whereas HLA class II molecules binds to the exogenous antigens found in intracellular endosomes and stimulates $CD4^+$ T cells (Parkin and Cohen 2001). For a proper peptide presentation via HLA molecules, native protein has to first degrade inside the APCs to a presentable length, a phenomenon referred as antigen processing.

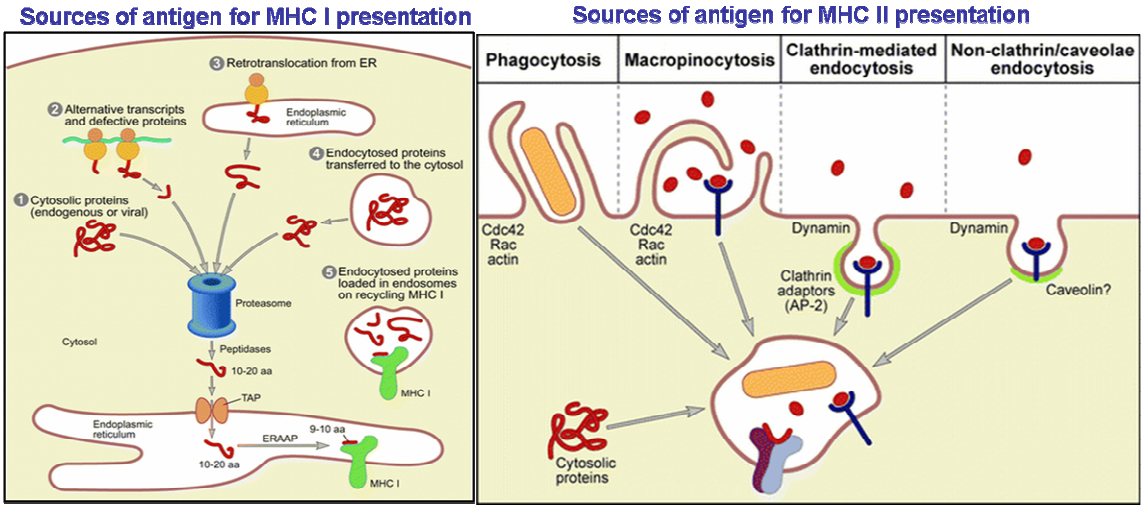


Figure 1: Sources of antigen for MHC class I and class II presentation. (Adapted from (Trombetta and Mellman 2005).

1.2.1 MHC/HLA class I antigen presentation

MHC class I molecule is present in all the nucleated cells and display proteasome processed protein antigens in the groove of MHC molecules. In humans, the HLA

complex is encoded by chromosome 6 and is extremely polymorphic (York and Rock 1996). In the absence of peptide antigen, it is stabilized by endoplasmic reticulum (ER) chaperone proteins calreticulin, ERp57, protein disulphide isomerase (PDI) and chaperone tapasin. Loading of peptides on the HLA molecules includes several steps. In the cytosol of APCs, proteins are first tagged by a multi-ubiquitin chain. These tagged proteins are recognized by immuno-proteasomes and degraded into smaller peptide sequences. The peptides are then transported into the ER lumen through the transporter associated with antigen presentation (TAP). Here the translocation is done with the help of tapasin, a TAP-associated glycoprotein. In ER, unfolded HLA class I molecules are assembled with the peptides and β2-microglobulin to form folded HLA class I molecule.

Once peptide binds to HLA molecule, tapasin is released and TAP is recycled for the next translocation of peptides to ER. In ER, there are three components which provide the stability to HLA class I molecule, a polymorphic heavy chain, a light chain (β2-microglobulin) and a peptide of eight to nine amino acids. This complex is now transported to the Golgi apparatus and then to the cell surface. In case of no assembly with peptides in ER, HLA molecules are recycled to the cytosol for degradation (Hughes, Hammond et al. 1997; Koopmann, Albring et al. 2000).

Due to low affinity of peptides, sometimes MHC class I heavy chains dissociate from peptides and then reassemble with exogenous peptides in the presence of serum β2-microglobulin (Rocca, Opolski et al. 1992). This effect is referred as cross-presentation where exogenous antigen peptides are loaded onto MHC class I molecules and presented to $CD8^+$ T cells (York and Rock 1996; Li, Haque et al. 2002; Rock 2006; Vyas, Van der Veen et al. 2008; Knecht, Aguado et al. 2009). This

phenomenon is very important in the initiation of an immune response when infection (either viral or bacterial) and cancer/leukaemia do not result in the generation of endogenous antigens.

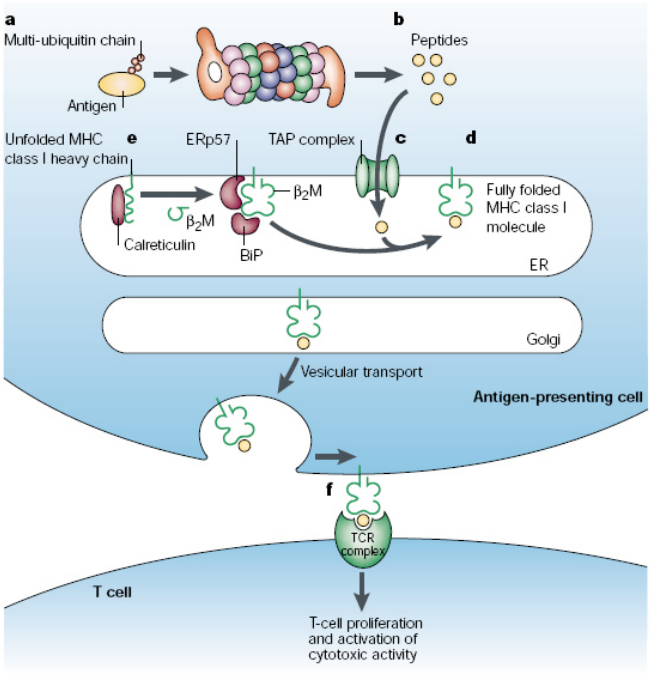


Figure 2: MHC/HLA class I processing and presentation. First step of antigen processing and presentation is the binding of multi-ubiquitin chain to the antigen. Antigen is then processed in proteasome which results in the generation of peptides of 8 to 11 amino acid residues. The peptides are transported into the endoplasmic reticulum (ER) by transporter associated with antigen presentation (TAP) complex. Within ER, peptides bind to MHC class I heterodimers and peptide-loaded MHC molecules are then translocated through Golgi apparatus to the cell surface. MHC class I molecule loaded with peptide binds specifically to complementary T cell receptors (TCRs) which lead to stimulation of $CD8^+$ T cells. **(Kloetzel 2001).**

In the groove of a MHC class I molecule, specificities of peptide loading are determined by anchor residues. One of the anchor residues is always present in the carboxy terminal of the post-proteasomal peptide. Another one or two could be present at the amino terminal (Falk, Rotzschke et al. 1991). For an adequate peptide

binding to a particular MHC class I molecule haplotype, the anchor residue must bind to the specific position defined to a particular haplotype.

1.2.2 Processing of VP1 protein, VLP

VLP is derived from one of the viral structural capsid protein VP1. The VP1 capsomere has the ability for self-assembly with the help of disulphide bonds to form the stable VLP (Chang, Fung et al. 1997; Chen, Wang et al. 2001). The assembly of VLP was first demonstrated after expressing VP1 protein in *E. coli* by Salunke et al. (Salunke, Caspar et al. 1986). VLP has been shown to display antigenic epitopes similar to the native virus. This property has been applied in early diagnosis of viral diseases using ELISA (Stolt, Sasnauskas et al. 2003; Viscidi, Rollison et al. 2003; Viscidi and Clayman 2006; Zielonka, Gedvilaite et al. 2006; Tolstov, Pastrana et al. 2009; Touze, Gaitan et al. 2010; Nicol, Touze et al. 2012; Zielonka, Gedvilaite et al. 2012; Nicol, Robinot et al. 2013) and hemagglutination assay (Zielonka, Gedvilaite et al. 2012). Additionally, the absence of genetic material in VLP makes it a safer alternative to be used as vaccine (Jennings and Bachmann 2008; Roldao, Mellado et al. 2010; Kushnir, Streatfield et al. 2012).

VLP has been shown to induce both humoral and cellular immune responses (Dorn, Lawatscheck et al. 2008; Jennings and Bachmann 2008). The size and the presence of natural ligands on the surface of VLP which are similar to the native virus help in the efficient uptake of VLP by the APCs, particularly by DCs. Other than professional APCs (DCs), peripheral blood mononuclear cells (PBMCs) loaded with JCV-VP1-VLP have also been used to stimulate T cells (Aly, Yousef et al. 2011).

1.3 Immunosuppression and viral infections

The human immune system is finely balanced to maintain the equilibrium between protection from pathogens and tolerance to self-antigens. There are several factors that can disturb this equilibrium and hence lower the efficiency of immune responses. For example, a state of immunosuppression, that leads to the reactivation of various latent viral or bacterial pathogens, which are otherwise controlled by the efficient immune system. Immunosuppression can be caused by malnutrition, ageing or infection and can also be induced by immunosuppressive agents.

In general, immunosuppression is used in the clinical setting to prevent the host from rejecting a transplant or for the treatment of autoimmune diseases. Immunosuppression is induced mainly by drugs or radiation. Under immunosuppression, opportunistic infections occur due to the reactivation of latent viruses in the patients or from the donor organs. Viral reactivation is one of the major causes of mortality and morbidity in most of the transplanted patients. The most common opportunistic viral pathogen which is reactivated in a transplantation setting is CMV. Other viral pathogens like EBV, adenovirus, herpes simplex virus (HSV), BKV, and JCV are also reported in a significant number of cases.

Besides induction through immunosuppressant agents and radiation, immunosuppression occurs in the state of malnutrition, ageing or caused by infections (HIV or other diseases). HIV related immunosuppression is characterized by a progressive depletion of $CD4^+$ helper T cells. This state of immunosuppression

is also a major cause for the reactivation of many latent viruses like CMV, EBV and JCV (Stoner, Agostini et al. 1998; Bienaime, Colson et al. 2006).

1.4 JC and BK polyomaviruses

Human polyomaviruses were first described in the 1950s, when they were identified as the causative agents of multiple tumors in rodents, hence the name polyoma ("poly" greek for many and the greek suffix "-oma" for tumors) was coined. Simian virus-40 (SV-40) was the first primate polyomavirus identified in African green monkey kidney cells (Sweet and Hilleman 1960). JC and BK polyomaviruses were later described in 1970s. JCV was isolated from brain tissue of a patient with the initials "JC" who suffered from PML (Padgett, Walker et al. 1971), and BKV grew in cell cultures after inoculation with the urine from a renal transplant recipient with the initials "BK" (Sweet and Hilleman 1960). Eight additional human polyomaviruses have been identified since 2007.

1.4.1 Morphology and genome

Polyomaviruses are small non-envelope particles encompassing the circular double-stranded deoxyribonucleic acid (DNA) genome. BKV and JCV genomes are approximately 5,300 bp and 5,130 bp in size, respectively (Ferenczy, Marshall et al. 2012). All members of the polyomavirus family share common morphology and genome organization. The polyomavirus genome can be divided into three functional parts (Figure 3).

i) The non-coding control region: contains viral promoters and the origin of replication.

ii) The viral early gene region which encodes the large T-antigen (LT-antigen) and the small T-antigen (sT-antigen). It is involved in the replication of viral genome.

iii) The viral late gene region which encodes the viral proteins VP1, VP2, VP3 as well as the agno-protein. It is involved in encoding structural proteins.

Among all the early and late gene expressing proteins, VP1 is the only protein exposed on the surface of the capsid and thus determines the receptor specificity.

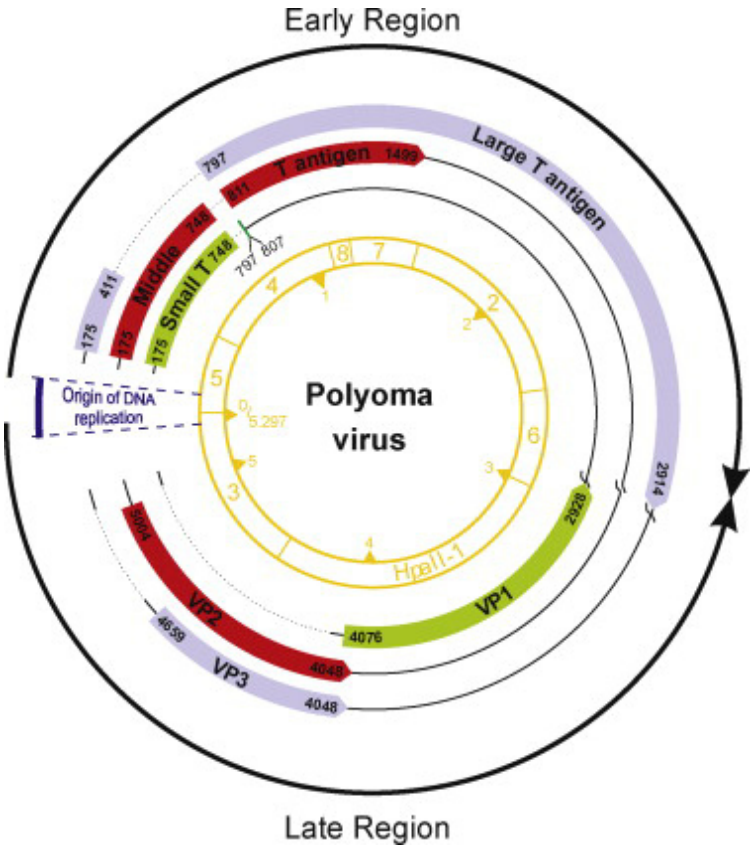


Figure 3: Organization of the genome of human polyomaviruses. (Atkin, Griffin et al. 2009).

1.4.2 Seroprevalence and infection

The asymptomatic primary contact with polyomaviruses occurs during childhood or early adulthood resulting in a lifelong persistence in immunocompetent individuals, leading to more than 80% seroprevalence in the adult population worldwide. Initial

infections of host cells are facilitated by the interactions of BKV and JCV with negatively charged sialic acid containing receptors. In the case of SV-40, ganglioside-monosialic acid (GM1) is the major receptor. In addition to sialic acid as receptor, the JC virus also uses the serotonin receptor ($5HT_{2A}R$) to infect glial cells (Elphick, Querbes et al. 2004). After primary infection, human polyomaviruses persist in the urinary tract, B lymphocytes and the brain (Chesters, Heritage et al. 1983).

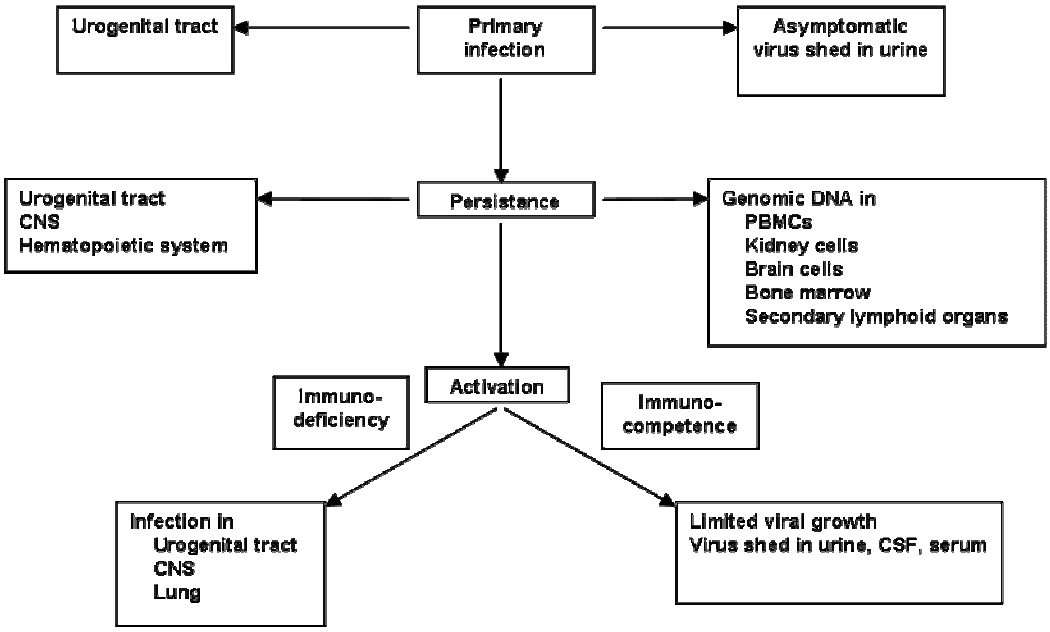


Figure 4: Clinical course of human polyomavirus infection. After primary infection JCV and BKV can persist in different types of tissue. In the immunocompetent host the polyomaviruses are controlled whereas immunodeficient patients are prone to clinically overt infection.

In case of JCV, infected B cells may carry virus across the blood brain barrier (BBB) (Major, Amemiya et al. 1990). Polymerase chain reaction (PCR) and antibody titer enzyme-linked immunosorbent assays (ELISA) confirm the presence of concomitant infections of JCV and BKV in HIV positive patients, post organ transplant patients, pregnant women and few healthy individuals. The pathophysiology of human polyomaviruses is explained in Figure 4.

1.4.3 JCV-associated complications and diagnosis

Unlike BKV, the other member of same genus, JCV is never involved in diseases of the urogenital tract or the lungs. The most prominent site of JCV-associated complications in the immunocompromised host is the central nervous system (CNS). Once JCV has entered the CNS, the virus replicates vigorously and leads to PML, a lethal disease. PML is a demyelinating disorder characterized by pinhead-sized demyelinating lesions in the brain. It occurs in 3-5% of HIV-infected patients, in patients suffering from autoimmune disorders, in cancer patients involving the lymphoid system (lymphoma), patients on long-term immunosuppressive therapy and few elderly patients with involution of the thymus. Previously, PML was considered as a rare opportunistic infection, but after the discovery of HIV, 80% of PML cases were contributed by HIV patients. The incidences of PML in non-HIV-infected patients are also increasing for hematologic malignancies (13%), organ transplant recipients (5%), and patients with autoimmune disease treated with immunomodulators (3%) (Gheuens, Pierone et al. 2010). In PML disease, tissue demyelination occurs due to the lytic activity of JCV in oligodendrocytes, which leads to the impairment of brain function and eventually to death. However, highly active antiretroviral therapy (HAART) in HIV patients has significantly improved the survival rate of PML patients (Berger and Houff 2006; De Luca, Ammassari et al. 2008). Nevertheless, the mortality associated with PML still approaches >50% within the first year (Tassie, Gasnault et al. 1999; Berenguer, Miralles et al. 2003).

The adverse effects of immunomodulatory therapy and immunosuppression are the major reason for immunosuppression leading to JCV-mediated fatal PML cases in patients. In immunosuppressed patients, immunomodulatory drugs like fludarabine,

methotrexate, cyclophosphamide and monoclonal antibodies like natalizumab (in multiple sclerosis and Crohn's disease), rituximab (in MS, non-HL, RA, SLE, MG diseases) etc. are responsible for inhibiting the biological function of immune cells. As anti-polyomavirus drugs with clinical efficacy are simply not available, the treatment solely relies on improving virus-specific immunity.

JCV causes PML during immunosuppressive conditions like the following.

Infections with HIV: The majority of the PML cases have been reported in HIV-1 patients. A very few cases are also reported in HIV-2 patients (Stoner, Agostini et al. 1998; Bienaime, Colson et al. 2006). With the advent of HIV treatment, the so-called HAART there was a significant improvement in the survival time of PML patients in HIV settings (Lewden, May et al. 2008). However, PML is still the second most common cause of death (14%) in HIV patients (Lewden, May et al. 2008).

In the setting of hematologic and oncologic malignancies: JCV-induced PML is also associated with lymho-proliferative disorders (Garcia-Suarez, de Miguel et al. 2005), like in chronic lymphocytic lymphoma, Hodgkin disease, non-Hodgkin lymphoma and multiple myeloma.

In the setting of stem cell and solid organ transplantations: JCV-reactivation occurs in transplantation setting due to the immunosuppressive conditions induced by intense immunosuppressive regimen (Shitrit, Lev et al. 2005; Kharfan-Dabaja, Ayala et al. 2007).

In patients with rheumatologic disorder: Systemic lupus erythematosus (SLE) is most commonly associated with JCV-induced PML. In other rheumatologic disorders like

rheumatoid arthritis, dermatomyositis, polymyositis and scleroderma the incidence of PML is rather low.

During monoclonal antibody (mAb) therapy: mAb treatment is practiced in many immunological diseases and few of these antibodies can suppress the immune system. For example, antibodies like, efalizumab, rituximab and natalizumab which are used in the setting of autoimmune diseases are associated with PML (Kleinschmidt-DeMasters and Tyler 2005; Langer-Gould, Atlas et al. 2005; Van Assche, Van Ranst et al. 2005; Carson, Focosi et al. 2009).

Other diseases: Few conditions of no or minimal immunodeficiency like renal failure, psoriasis, dermatomyositis etc. are also reported to cause immunosuppression and make patients prone to PML (Gheuens, Pierone et al. 2010).

In PML patients, MRI scans of the brain show the characteristic multifocal areas of demyelinating white matter lesions with irregular borders. Other than MRI scans, an indirect way of diagnosis is to detect JCV-DNA in the cerebrospinal fluid (CSF) by PCR amplification. However, PCR analysis is less sensitive in HIV patients receiving HAART (Cinque, Koralnik et al. 2009).

1.4.4 Adaptive immune responses to the JCV

1.4.4.1 Humoral immune response

Humoral immune responses are mediated by certain macromolecules such as secreted antibodies and complement proteins. Antibodies are soluble protein molecules secreted by B cells that are highly specific to foreign targets called antigens. As adults are already exposed to polyomaviruses during their childhood, most of them are seropositive for polyomavirus-specific serum antibodies. In most of

the viral infections, antibodies serve as neutralizing agents targeting viral proteins and facilitate the elimination of viruses by phagocytic cells. In polyomavirus-reactive patients, elevated antibody titers are seen only after the decrease of viral load (Comoli, Azzi et al. 2004; Hariharan, Cohen et al. 2005; Chen, Trofe et al. 2006), which suggests a role of $CD4^+$ T helper cells in stimulating B cells to differentiate into plasma cells. These antibodies are not effective in controling the polyomavirus-associated complications, but they may be effective in controling viremia.

Approximately 60-90% of the world population is seropositive to JCV and 19-27% of healthy individuals shed JC virions in the urine (Walker and Padgett 1983; Markowitz, Thompson et al. 1993; Egli, Infanti et al. 2009). Although the majority of the population is positive, a very small fraction of these individuals progress to PML disease mostly due to immunosuppressive changes in their immune system. Overall, the JCV-specific humoral immune response has not been shown to control JCV-infection effectively (Weber, Goldmann et al. 2001; Koralnik 2002). Also the nature of JCV to remain intra-nuclear after viral assembly seems to help in the viral escape from immune recognition.

1.4.4.2 Cellular immune response

Cellular immunity, the other branch of adaptive immunity comes in play when infectious virions enter host cells. Polyomavirus-specific responses mostly belong to this branch of the immune system, comprising extensively studied T cells. Studies in a mouse model of polyomavirus infection (Lukacher and Wilson 1998) and as well as observations in patients showed that the cytotoxic T lymphocyte (CTL) response has a strong correlation to disease outcome in the case of both JCV and BKV (Li, Melenhorst et al. 2006; Binggeli, Egli et al. 2007).

$CD8^+$ T cells (Du Pasquier, Clark et al. 2001; Koralnik, Du Pasquier et al. 2001; Wuthrich, Kesari et al. 2006; Lima, Marzocchetti et al. 2007; Lima, Bernal-Cano et al. 2010) as well as $CD4^+$ T cells (Gasnault, Kahraman et al. 2003; Aly, Yousef et al. 2011) have been shown to be effective in the control of JCV infection. The advent of HAART in HIV patients reduced the rate of PML which indirectly strengthens the overall immune response by reconstituting T cell responses (Albrecht, Hoffmann et al. 1998; Engsig, Hansen et al. 2009). In these patients, HAART has no direct effect on the replication of JCV but seems to be effective against JCV by limiting HIV replication.

1.4.5 Treatment of polyomavirus disease

So far, therapeutic approaches for PML have shown less or almost no significant clinical impact (Tavazzi, White et al. 2012). This could be due to the BBB which makes the CNS less accessible to chemical drugs. Nevertheless, a few clinical studies showed an improvement of clinical neurological symptoms and findings in the MRI after administration of risperidone (Focosi, Kast et al. 2007), mitrazapine (Owczarczyk, Hilker et al. 2007; Verma, Cikurel et al. 2007; Cettomai and McArthur 2009) and recombinant IL-2 (Przepiorka, Jaeckle et al. 1997; Kunschner and Scott 2005). One drug, cidofovir showed clinical impact in BKV-induced HC patients, however no significant clinical effect was reported on the survival of PML patients (Marra, Rajicic et al. 2002; Pohlmann, Hochauf et al. 2007; De Luca, Ammassari et al. 2008; Kraemer, Evers et al. 2008). In contrast to that, an immunotherapeutic approach showed some promise: Here a patient with the clinical status of JCV-associated PML greatly improved and showed a complete clearance of JCV-DNA in the cerebrospinal fluid after the adoptive transfer of JCV-specific *in vitro* activated

CTLs (Balduzzi, Lucchini et al. 2011). In summary, there are no clear guidelines for the drug treatment of JCV-related infection or reactivation and successful therapy depends greatly on the overall improvement of immune system. For example, in patients with PML, HAART improves the overall immune system hence it is the only therapy which seems to have an effective clinical outcomes.

1.5 Adoptive immunotherapy

Viral infection during immunosuppression is a major complication in transplantation, rheumatologic, and other immunodeficient condition (HIV). For the successful eradication of viral infection, anti-viral therapies are a pre-requisite. Anti-viral therapies post immunosuppression falls into 3 strategic categories:

i) A prophylactic therapy, also called as preventive therapy is administrated before an active viral infection occurs.

ii) A pre-emptive therapy applied at the stage of infection with sensitive and rapid monitoring methods while still no disease could be diagnosed.

iii) A curative therapy applied after establishment of viral disease. This comprises mostly anti-viral drug therapy.

Anti-viral drugs are effective to combat viral infection or reactivation. However, these drugs are not very effective in the total elimination of latent viral reservoir and the impact on the overall survival of patients is still questioned. Moreover, due to the disadvantages like costs, toxicity, drug-resistant mutants and the risk ofr secondary infections, there is a fervent need of other therapeutic strategies/approaches.

Immunotherapy is one effective approach which is defined as the treatment of disease by manipulating the immune system of patients (Figure 5).

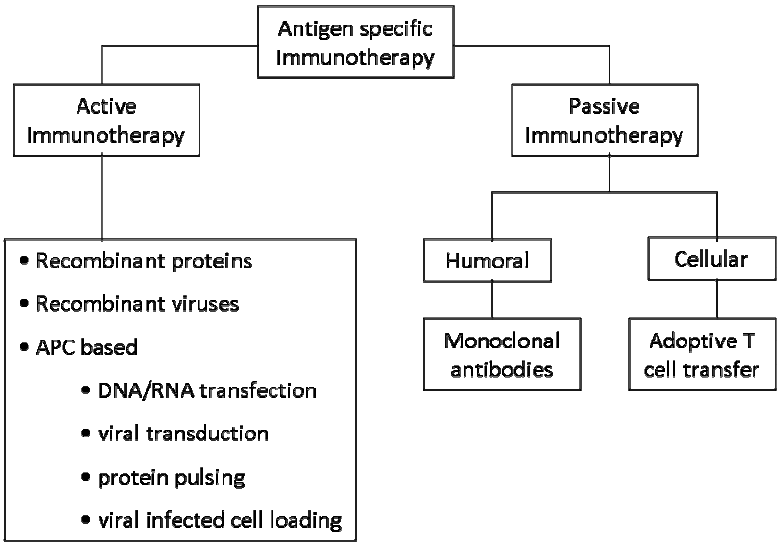


Figure 5: Overview of antigen-specific immunotherapeutic strategies. (Adapted from (Van den Bosch, Ponsaerts et al. 2006)

Immunotherapeutic approaches have been evolved from infusion of non-antigen-specific T cells, a donor lymphocyte infusion (DLI) to the selection and isolation of antigen-specific T cells during the past few decades. During DLI, a large number of donor T cells are also transfused into the host. Although these mature T cells are responsible for the protection against infectious diseases, they are also largely responsible for the unintended graft versus host disease (GvHD). To overcome this problem one option is to reconstitute the immune system by the adoptive transfer of donor T cells. This should be performed after few weeks or months from transplantation, in order to reduce the risk of GvHD. Moreover, when adoptive transfer is performed using peptide epitope-specific T cells the risk of GvHD is almost nil.

Study by Riddell and Greenberg's group showed for the first time a successful adoptive transfer of CMV-specific T cells in the setting of hematopoietic stem cell transplantation (HSCT) (Riddell, Watanabe et al. 1992). Here T cell lines were transfused between 28 and 42 days after transplantation. A highly CMV-specific cytotoxicity was observed in all patients in this study, and in few patients it reached the same as the donor. Some other studies also showed the successful adoptive transfer of CMV (Walter, Greenberg et al. 1995; Einsele, Roosnek et al. 2002; Peggs, Verfuerth et al. 2003) and EBV (Papadopoulos, Ladanyi et al. 1994; Rooney, Smith et al. 1995; Rooney, Smith et al. 1998; Haque, Wilkie et al. 2002)-specific T cells after transplantation.

Progress in flow cytometry and the advent of multimers facilitates monitoring and isolation of viral-specific T cells with high purity and either directly from PBMCs or expanded T cells. A study by Cwynarski et al. showed for the first time quantification of reconstituted CMV-specific T cells by MHC-tetramers in patients after allogeneic-SCT (Cwynarski, Ainsworth et al. 2001). In a classical pioneer study, Cobbold et al. demonstrated enhanced immune reconstitution and clearance of the infection in 8 out of 9 patients who had undergone transfer of MHC-tetramer/magnetically isolated CMV-specific $CD8^+$ T cells (Cobbold, Khan et al. 2005).

More recently, both the introduction of multimers (like streptamers, pentamers etc.) and the IFN-γ capture assay facilitated the isolation of good manufacturing practice (GMP) grade virus-specific T cells. Some studies showed the successful isolation by IFN-γ capture assay and consecutive transfusion of functional T cells specific for CMV (Feuchtinger, Opherk et al. 2010; Peggs, Thomson et al. 2011), EBV (Moosmann, Bigalke et al. 2010) and adenovirus (Feuchtinger, Richard et al. 2008)

in the clinical setting. Schmitt et al. showed for the first time CMV-specific adoptive immunotherapy using streptamer isolation (Schmitt, Tonn et al. 2011). In humans, adoptive immunotherapy also includes administration of TCR-transfected PBMCs. In this context, Yang *et al.* generated TCR-transfected anti-JCV T cells (specific to VP1, p36 and p100; two HLA-A2 restricted immunodominant peptides) (Yang, Beaudoin et al. 2007).

In the case of JCV-associated infections, a single case study using JCV-specific T cells as adoptive T cell therapy has been reported (Balduzzi, Lucchini et al. 2011). Here, JCV-stimulated T cells were not single-epitope-specific rather, PBMCs were expanded using 15mer peptide pool of entire JCV-VP1 and LT-antigen proteins.

For an effective antigen-specific immunotherapeutic strategy, the knowledge of HLA-restriction and selection of a potent immunogenic peptide antigen is the most critical step. In this context, the availability of overlapping matrix peptide pool is an excellent tool for the selection of immunodominant epitopes across the viral genome. As a different approach, advanced knowledge about HLA class I and II gives an added advantage to use more sophisticated strategy to identify HLA-binding motifs (discussed in 1.8.1.3). This latter bioinformatics-based prediction approach follows excellent prediction protocols but needs an experimental confirmation of epitope immunogenicity. By employing one or both approaches, some BKV and JCV-specific peptide epitopes have been mentioned in earlier reports (Table 1)

Table 1: Studies reporting BKV and JCV-specific HLA-A2 restricted peptide sequences. (#HLA-B0702 and B08 restricted).

BKV/JCV peptide sequence	References
BKV-VP1-p44 AITEVECFL JCV-VP1-p36 SITEVECFL	Chen, Y., et al (2006); Lima, M.A., et al (2007); Sharma, M.C., et al (2006); van Aalderen, M.C., et al (2013); Du Pasquier, R.A., et al (2004); Zhou, W., et al (2007); Yang, W., et al (2007); Marzocchetti, A., et al (2009); Du Pasquier, R.A., et al (2006); Du Pasquier, R.A., et al (2005); Tan, C.S., et al (2013)
BKV-VP1-p108 LLMWEAVTV JCV-VP1-p100 ILMWEAVTL	Chen, Y., et al (2006); Lima, M.A., et al (2007); Sharma, M.C., et al (2006); van Aalderen, M.C., et al (2013); Du Pasquier, R.A., et al (2004); Zhou, W., et al (2007); Schneidawind, D.,et al (2010); Yang, W., et al (2007); Marzocchetti, A., et al (2009); Du Pasquier, R.A., et al (2006); Du Pasquier, R.A., et al (2005); Tan, C.S., et al (2013); Koralnik, I.J., (2002); Yang, W., et al (2006); Koralnik, I.J., et al (2002)
BKV-T-p579 LLLIWFRPV	Randhawa, P.S., et al (2006); Provenzano, M., et al (2006); van Aalderen, M.C., et al (2013); Zhou, W., et al (2007); Chen, Y., et al (2008)
BKV-T-p570 ILQSGMTLL	Provenzano, M., et al (2006); Chen, Y., et al (2008)
BKV-T-p398 CLLPKMDSV	Provenzano, M., et al (2006); Chen, Y., et al (2008)
BKV-T-p410 FLHCIVFNV	Randhawa, P.S., et al (2006); Provenzano, M., et al (2006); van Aalderen, M.C., et al (2013); Chen, Y., et al (2008)
BKV-T-p362–371 MLTERFNHIL	Randhawa, P.S., et al (2006); Provenzano, M., et al (2006); Zhou, W., et al (2007)
BKV-T-p406–414 VIFDFLHCI	Randhawa, P.S., et al (2006); Provenzano, M., et al (2006); Zhou, W., et al (2007)
BKV-VP1-p27 LLIKGGVEV	Chen, Y., et al (2006); Schneidawind, D.,et al (2010)
BKV-VP1-p245 LLDEQGVGPL	Schneidawind, D.,et al (2010)
BKV-VP1-p62 NLRGFSLKL	Schneidawind, D.,et al (2010)
BKV-VP1-p90 STARIPLPNL	Schneidawind, D.,et al (2010)
BKV-T-p10 SMELMDLLGL	Schneidawind, D.,et al (2010)
BKV-T-p125 FLRKEPLVWI	Schneidawind, D.,et al (2010)
BKV-T-p4 VLNREESME JCV-T-p4 VLNREESME	Schneidawind, D.,et al (2010)
BKV-VP1-p84 KMLPCYSTA	Chen, Y., et al (2006)
BKV-VP1-p128 NLHAGSQKV	Chen, Y., et al (2006)
BKV-VP1-p259 SLYVSAADI	Chen, Y., et al (2006)
BKV-T-p313 PYHFKYHEKHFANAI	Ramaswami, B., et al (2011)
BKV-T-p216 KLCTFSFLI	Provenzano, M., et al (2006)
BKV-T-p199 FLTPHRHRV	Provenzano, M., et al (2006)
BKV-T-p176 KLMEKYSVT	Provenzano, M., et al (2006)
BKV-T-p157 TLACFAVYT	Provenzano, M., et al (2006)
BKV-T-p558 SLQNSEFLL	Provenzano, M., et al (2006)
BKV-T-p395 WLHCLLPKM	Provenzano, M., et al (2006)
BKV-T-p436 TLAAGLLDL	Provenzano, M., et al (2006)
BKV-T-p472 VVFEDVKGT	Provenzano, M., et al (2006)
# BKV-T-antigen LPLMRKAYL # JCV-T-antigen IPVMRKAYL	Li, J., et al (2006)

1.6 Characterization of novel T cell epitopes (TCEs)

T cell epitope (TCE) is a peptide sequence which is derived from an antigen, binds to HLA molecule and can elicit an immunogenic T cell response. In order to be an authentic $CD8^+$ TCE, peptide has to be naturally processed inside the APCs. Epitope mapping is a process of identifying T cell determinants or TCE-specific to particular HLA restricted peptides from a relevant protein of pathogen. It is a crucial step in the development of peptide or T cell-based immunotherapies.

1.6.1 Strategies for T cell mapping

1.6.1.1 Overlapping peptides

In order to map all the epitopes derived from the protein of a potential pathogen, peptides of 15-18mers overlapped by 11 amino acids are most commonly used. Due to the overlapping of amino acid sequences, it has an advantage of not missing any antigenic determinants or epitopes. Although, it is well known that the peptide length of 8 to 10 amino acids can stimulate an optimal $CD8^+$ T cell responses, 15mer peptides are also shown to trigger $CD8^+$ T cell responses in a comparable manner (Kiecker, Streitz et al. 2004).

1.6.1.2 Peptide matrices

To save the samples or cells, peptide matrices have been introduced (Kern, Bunde et al. 2002). Peptide matrix contains grid of peptide sub-pools arranged horizontally and vertically (Figure 13). In a matrix pool each peptide is contained in two different peptide sub-pools. So when a particular peptide is recognized in the whole set, two peptide sub-pools containing the same peptide will be positive. In case, when more than two sub-pools are positive, there is a need of further testing to recognize the

targeted peptide. Peptide matrix approach is particularly useful when the cell number is limited.

1.6.1.3 *In silico* approach

Many computer algorithm software programs have been developed for the prediction of MHC class I and II epitope peptides in the last two decades (Roberts, Meister et al. 1996; Rammensee, Bachmann et al. 1999; De Groot, Bosma et al. 2001; Martin, Sbai et al. 2003; Bui, Schiewe et al. 2006). These prediction programs are based on binding affinity, cleavage sites and TAP-binding peptides. Prediction programs are faster, cheaper and can be used to delete non-HLA binding peptides thus increasing the possibilities of potential epitopes in a peptide library. Since algorithm protocols are based on assumptions and because of the complexity and vastness of HLA molecule, *in silico* predicted epitopes should be also experimentally confirmed in *in vitro* "wet bench" experiments.

1.6.2 Methods used for T cell epitope mapping:

a) **HLA-peptide multimers**: Multimers are complexes made of specific HLA class I or class II alleles bound to a specific peptide. Additionally, this construct can be visualized in flow cytometry when tagged with a fluorochrome. Multimers are easy-to-use tools and precise. They can be combined with many other phenotypic or intracellular markers for fine characterization of peptide-specific T cells. In order to assign multimers for epitope mapping, prior knowledge of HLA-binding peptides is necessary. This makes it difficult to use multimers for the identification of new epitopes. To this direction, a novel approach based on reversible peptide-binding on HLA class

I allele was proposed for epitope mapping (Toebes, Coccoris et al. 2006). Here, HLA-allele with peptide is first dissociated using ultra-violet (UV) and then competitively replaced by a second new peptide with the same HLA-binding capability but of different specificity. Thus, a new specificity can be recognized from a set of peptides. Although, the approach of MHC-peptide multimers is attractive, the availability of few HLA-alleles limits its use for all known human haplotypes.

b) **Enzyme-linked immunospot (ELISPOT)**: ELISPOT is based on the detection of secreted cytokines or any other effector molecules (e.g. granzyme B) when T cells are stimulated specifically or non-specifically. This procedure can be used for the measurement of the clonal size and the functional property of low-frequency antigen-specific T cells. In an ELISPOT assay, a single dot represents one cytokine-secreting cell. Thus it is easy to quantify the number of cells secreting a given cytokine. It is also possible to simultaneously detect two different cytokines from a single cell using different detection reagents (Wolfl, Kuball et al. 2007). ELISPOT is easy, requires simple instrumentation and is widely used for epitope mapping.

c) **Intracytoplasmic cytokine staining (ICS)**: In principle, this approach is similar to that of cytokine secretion assessed in ELISPOT assays. However, here the cytokines are retained inside the cell using a reagent which inhibits the secretion of cytokines from Golgi apparatus outside the cell. After permeabilization with a detergent, trapped cytokines are stained with fluorochrome-conjugated antibodies and visualized by flow cytometry. The

advantage of ICS is that it can be combined with many phenotypic markers or other secreting cytokines in a multiparametric flow cytometric setting.

d) **Lympho-proliferation**: Lympho-proliferation relies on the proliferation of antigen-specific T cells. In this context, radioactive like 3H-thymidine uptake assay and non-radioactive like carboxyfluorescein succinimidyl ester (CFSE) constitute commonly used approaches for epitope mapping. Since lympho-proliferation assays are not indicators for the functional aspects of proliferative cells, this approach has been mostly used for initial pre-screening or has been combined with ICS for epitope mapping (Tesfa, Volk et al. 2003).

e) **Solid phase HLA-peptide complexes**: It constitutes an approach in which solid phase HLA-peptide complexes can be used to recognize and select specific T cells.

f) **Other methods**: Methods like cytokine-capture/secretion assay or measurement of activation markers (CD154, CD137 etc.) on antigen-specific activated T cells are also used as an epitope mapping strategy.

2 MATERIAL AND METHODS

2.1 MATERIAL

2.1.1 Plasticware

BD Falcon®, polystyrene, round-bottom tube	Cat. No. 352054, BD biosciences, Heidelberg, Germany
CELLSTAR® 24 well culture plate	Cat. No. 662160, Greiner Bio-One, Frickenhausen, Germany
CELLSTAR® polypropylene tubes, 50 ml	Cat. No. 227 261, Greiner Bio-One, Frickenhausen, Germany
CELLSTAR® polypropylene tubes, 15 ml	Cat. No. 188 271, Greiner Bio-One, Frickenhausen, Germany
CELLSTAR® serological pipettes, 2 ml	Cat. No. 710 180, Greiner Bio-One, Frickenhausen, Germany
COMBITIPS BIOPUR plus, 5.0 ml	Cat. No. 0030.069.455, Eppendorf, Hamburg, Germany
Cryos™ freezing tubes, 2 ml	Cat.-No.122277, Greiner Bio-One, Frickenhausen, Germany
Multiply µstrip (PCR tubes)	Cat.-No. 72.991.002, Sarstedt, Nümbrecht, Germany
Disposable protective gloves, M	Sempermed, Wien, Austria
Disposable-scalpel	Cat. No. 5205052, Feather, Japan
Eppendorf T.I.P.S. standard 50-1000 µl	Cat. No. 0030 000.919, Eppendorf, Hamburg, Germany
High-quality pipette tips in bags, racks, and space-saving system packaging, 200 µl	Cat. No. 70.1130.600, Sarstedt, Nümbrecht, Germany
High-quality pipette tips in bags, racks, and space-saving system packaging, 10 µl	Cat. No. 70.760.501, Sarstedt, Nümbrecht, Germany
MS columns	Cat. No.130-042-201, Miltenyi Biotec, Bergisch - Gladbach, Germany
Multi-screen-IP, 0.45 µm, clear, sterile	Cat. No.MAIPS4510, Millipore, Darmstadt, Germany
Nunclon™ treated flask, 75 cm^2	Cat. No. EW-01930-49, Nunc, Denmark
Pasteur capillary pipette, 150 mm and 230 mm	W.U. Mainz, Germany
Petri dish, round, with 2 compartments	Cat. No. 82.1195, Sarstedt, Nümbrecht, Germany
Pre-separation filter, 30 µm	Cat. No. 130-041-407, Miltenyi Biotec, Bergisch - Gladbach, Germany
Serological pipettes, 5 ml	Cat. No. 86.1253.001, Sarstedt, Nümbrecht, Germany
Serological pipettes, 10 ml	Cat. No. 86.1254.001, Sarstedt, Nümbrecht, Germany
Serological pipettes, 50 ml	Cat. No. 86.1685.001, Sarstedt, Nümbrecht, Germany
Tips without filter, 1000 µl	Cat. No. 70.762.200, Sarstedt, Nümbrecht, Germany
Transfer pipette, 3.5 ml	Cat. No. 86.1171.001, Sarstedt, Nümbrecht, Germany
Tube for FACScan flow cytometer 5 ml (75x12 mm, PP)	Cat. No. 551578, Sarstedt, Nümbrecht, Germany

2.1.2 Medium, Solutions and buffers

autoMACS rinsing solution	Cat No. 130-091-222, Miltenyi Biotec, Bergisch - Gladbach, Germany
Cell staining buffer	Cat No. 420201, Biolegend, San Diego, CA, USA
Distilled water	Cat No. 10977-035, Gibco, Grandisland, NY. USA
DPBS (1X)	Cat No. 14190-169, Gibco, Grandisland, NY. USA
Fetal bovine serum "GOLD"	Cat. No. A15-151, PAA, Linz, Austria
FicoLite-H (Human); Sterile filtered	Cat No. GTF1511YK, LINARIS, Dossenheim, Germany
MACS BSA stock solution	Cat No. 130-091-376, Miltenyi Biotec, Bergisch - Gladbach, Germany
RPMI medium 1640 (1X)	Cat No. 21875-034, Gibco, Grandisland, NY. USA
Trypan blue solution	Cat No. T8154-20ML, Sigma Aldrich, Steinheim, Germany
Complete RPMI1640 medium	Containing 1% penicillin/streptomycin,1% L-glutamine and 10% fetal bovine serum

2.1.3 Solutions, buffers and antibodies for ELISPOT

BCIP®/NBT liquid substrate system	Cat No. B1911-100ML, Sigma Aldrich, Steinheim, Germany
BD™ ELISPOT AEC substrate set	Cat No. 551951, BD Biosciences, Heidelberg, Germany
AEC substrate	Cat No. 51-2577KC, BD Biosciences, Heidelberg, Germany
AEC chromogen	Cat No. 51-2578KC, BD Biosciences, Heidelberg, Germany
Blocking solution for granzyme B	RPMI1640 complete culture medium
Blocking solution for INF-γ	PBS + 10 % FBS
Human granzyme B ELISPOT set	Cat No. 552572, BD Biosciences, Heidelberg, Germany
Granzyme B capture-antibody	Cat No. 51-9000058, BD Biosciences, Heidelberg, Germany
Granzyme B detection-antibody	Cat No. 51-900060, BD Biosciences, Heidelberg, Germany
Granzyme B streptavidin-HRP	Cat No. 51-9000209, BD Biosciences, Heidelberg, Germany
Human IFN-γ ELISpot kit (ALP)	Cat No. 3420-2A, Mabtech AB, USA
Human IFN-γ coating mAb 1-D1K; purified, 250 µg	Cat No. 3420-3-250, Mabtech AB, USA
Human IFN-γ detection mAb (7-B6-1-biotin)	Cat No. 3420-6-250, Mabtech AB, USA
Streptavidin ALP (alkaline phosphatase)	Cat No. 3310-8, Mabtech AB, USA

2.1.4 Antibodies and tetramers for flow cytometry

APC anti-human CD137 (4-1BB) antibody	Cat No. 309810, Biolegend, San Diego, CA, USA
APC anti-human CD8 (SK1) antibody	Cat No. 344722, Biolegend, San Diego, CA, USA
APC mouse anti-Human IFN-γ	Cat No. 554702, BD biosciences, Heidelberg, Germany
APC mouse IgG1, κ isotype control	Cat No. 554681, BD biosciences, Heidelberg, Germany
CMVpp65 MHC-tetramer APC	HLA-A*0201 tetramers CMVpp65 495-503 (NLVPMVATV) APC, LICR, Lausanne, Switzerland
CMVpp65 MHC-tetramer PE	HLA-A*0201 tetramers CMVpp65 495-503 (NLVPMVATV) PE, LICR, Lausanne, Switzerland
JCV-VP1-p100 MHC-tetramer PE	HLA-A*0201 tetramers JCV-VP1-p100-108 (ILMWEAVTL) PE, LICR, Lausanne, Switzerland
JCV-VP1-p36 MHC-tetramer PE	HLA-A*0201 tetramers JCV-VP1-p36-P44 (SITEVECFL) PE, LICR, Lausanne, Switzerland
BKV-VP1-p108 MHC-tetramer PE	HLA-A*0201 tetramers BKV-VP1-p108-117 (LLMWEAVTV) PE, LICR, Lausanne, Switzerland
FITC anti-human CD8 (SK1) antibody	Cat No. 345772, BD biosciences, Heidelberg, Germany
FITC anti-human HLA-A2 (BB7.2) antibody	Cat No. 343304, Biolegend, San Diego, CA, USA
FITC mouse IgG1, κ isotype control	Cat No. 555748, BD biosciences, Heidelberg, Germany
PE anti-human CD137 (4-1BB) antibody	Cat No. 309804, Biolegend, San Diego, CA, USA
PE mouse IgG1, κ isotype control	Cat No. 555749, BD biosciences, Heidelberg, Germany
Unconjugated HLA-A1, A36 antibody	Cat No. 0289HA, One lambda, CA, USA
FITC, HLA-B7, 27 antibody	Cat No. FH1453, One lambda, CA, USA
FITC, HLA-B8 antibody	Cat No. FH0536A, One lambda, CA, USA

2.1.5 Reagents and chemicals

Autopure 100% isopropanol reagent	Cat No. 949016, QIAGEN, Maryland, USA
CD8 microbeads, human	Cat No. 130-045-201 Miltenyi Biotec, Bergisch-Gladbach, Germany
Dimethyl sulfoxide	Cat No. D5879-100ML Sigma Aldrich, Steinheim, Germany
di-Sodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2\ H_2O$)	Cat No. 4984.1, Carl Roth, Karlsruhe, Germany
Ethanol absolute	Cat No. 32205-1L, Sigma Aldrich, Steinheim, Germany
Human IL-2 (5 µg)	Cat No. 130-093-901, Miltenyi Biotec, Bergisch-Gladbach, Germany
Human IL-7 (10 µg)	Cat No. 130-093-937, Miltenyi Biotec, Bergisch-Gladbach, Germany
L-alanyl-L-glutamine (200 mM)	Cat No. 0638 W, Biochrom AG, Berlin
Natriumchloride (NaCl)	Cat No. 3957.1, Carl Roth, Karlsruhe, Germany
Paraformaldehyde	Cat No. 158127-100G, Sigma Aldrich, Steinheim, Germany

Penicillin/streptomycin	Cat No. A2213, Biochrom AG, Berlin
Phytohemagglutinin	Cat No. L9017-5MG, Sigma Aldrich, Steinheim, Germany
Saponin	Cat No. 47036-50G-F, Sigma Aldrich, Steinheim, Germany
Sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$)	Cat No. K300.2, Carl Roth, Karlsruhe, Germany
Staphylococus entertoxin B	Cat No. S9008-1VL Sigma Aldrich, Steinheim, Germany
TRIS	Cat No. 4835.2, Carl Roth, Karlsruhe, Germany
Tween20® detergent, 100ml	Cat No. 655204, Merck, Darmstadt, Germany
Agarose	Cat. No. A8963, Applichem, Gatersleben, Germany
Ethidium bromide 0.025%	Cat.-No. HP47.1, Roth, Karlsruhe, Germany
TrackIt™ DNA ladder	Cat.-No. 10488-058, Invitrogen, California, USA
6x orange DNA loading dye	Cat.-No. R0631, Thermo scientific, Wisconsin, USA
AmpliTaq DNA polymerase	Cat. No. M05768, Roche, Basel, Switzerland
10x PCR Buffer	Cat. No. KP2037, Roche, Basel, Switzerland

2.1.6 Equipments

-80°C Ultra low temperature freezer	SANYO Electric biomedical co. ltd, Japan
Aqualine AL 12	LAUDA, Germany
Autoclave, Systec VX-150	Systec GmbH, Wettenberg, Germany
BD FACScan™ Flow Cytometer	BD biosciences, New Jersey, USA
Elispot reader	CTL, Bonn, Germany
Hemocytometer chamber	Optik labor, Berlin, Germany
Heraeus megafuge 16 centrifuge	Thermo scientific, Wisconsin, USA
Heracell 150i CO_2 incubators	Thermo scientific, Wisconsin, USA
Herasafe KS, Class II biological safety cabinet	Thermo scientific, Wisconsin, USA
Inverse microscope	Hund Wetzlar, Wetzlar, Germany
KERN EG 2200-2NM	KERN & Sohn GmbH, Balingen, Germany
Light microscope	Hund Wetzlar, Wetzlar, Germany
Liquid nitrogen tank	Model 8038 S/N 14830.59 Forma scientific Inc. Germany
Magnetic stirrer	Ikamag TRC, Renner GmbH, Ludwigshafen, Germany
Microcentrifuge 5424R	Eppendorf, Hamburg, Germany
MiniMACS™, MultiStand	Miltenyi Biotec, Bergisch-Gladbach, Germany
Mixter, Reax top	Heidolph Instruments GmbH & Co. KG, Schwabach, Germany
Mr. Frosty freezing container	Thermo scientific, Wisconsin, USA
Multipette® stream	Eppendorf, Hamburg, Germany
pipetus® Pipette boy	Hirschmann Laborgeräte, Eberstadt Germany
Refrigerator	Liebherr, Ochsenhausen, Gemany
Research® plus 3-packs	Eppendorf, Hamburg, Germany
Research® plus (fixed) 0.1-2.5 µl	Eppendorf, Hamburg, Germany
Research® plus (multichannel) 30-300 µl	Eppendorf, Hamburg, Germany
VACUSAFE (158 320)	INTEGRA Biosciences AG, Zizers, Switzerland

2720 Thermal cycler	Applied Biosystems, California, USA
PTC0200 DNA engine gradient thermal cycler	MJ research, Inc., Waltham, Massachusetts, USA
Electrophoresis gel apparatus	PEQLAB Biotechnologie GmbH, Germany
Bio doc analyzer	Biometra, Goettingen, Germany
Electrophoresis power supply (EU231)	PEQLAB Biotechnologie GmbH, Germany
Nanodrop 2000c	Thermo scientific, Wisconsin, USA

2.1.7 Primers

HLA-A2	5'-GTGGATAGAGCAGGAGGGT-3' (forward) 5'-CCAAGAGCGCAGGTCCTCT-3' (reverse)
HLA-B8	5'-GACCGGAACACACAGATCTT -3' (forward) 5'-CCGCGCGCTCCAGCGTG-3' (reverse)
HLA-B7	5'-CAAGTGGGAGGCGGCCCGTGA-3' (forward) 5'-TGGTACCAGCGCGCTCCAGCT-3' (reverse)
HLA-A1	5'-GCGGACATGGCAGCTCAGAT-3' (forward) 5'-CGGAGCCCGTCCACGCACC-3' (reverse)
CXADR gene (exon 2)	5'-CTGGGCATCTCTTGAGTTTGGA-3' (forward) 5'-ACTGGCAAGGTGATGGACACAT-3'(reverse)
Human β-actin	5'-CTGGGACGACATGGAGAAAA-3' (forward) 5'-AAGGAAGGCTGGAAGAGTGC-3' (reverse)
Mycoplasma	5'-GGCGAATGGGTGAGTAACACG-3' (forward) 5'-CGGATAACGCTTGCGACCTATG-3' (reverse)

2.1.8 Peptides, peptide pool and proteins

All the peptides cited or identified from the protein sequences were designated according to the position of starting amino acid (e.g. JCV-VP1-p36-44 as p36). All the overlapping peptides were designated with the prefix OP (e.g. JCV-VP1 overlapping peptide 1 as OP1). JCV and BKV-VLP were a kind gift from Prof. Alma Gedvilaite (Vilnius University).

JCV-VP1-p36-44, SITEVECFL	GL Biochem (Shanghai) Ltd.; Minhang Shanghai and DKFZ, Heidelberg, Germany
JCV-VP1-p100-108, ILMWEAVTL	GL Biochem (Shanghai) Ltd.; Minhang Shanghai and DKFZ, Heidelberg, Germany
BKV-VP1-p108-117, LLMWEAVTV	GL Biochem (Shanghai) Ltd.; Minhang Shanghai
JCV-VP1-p12-20, DPVQVPKLL	DKFZ, Heidelberg, Germany
JCV-VP1-p78-p86, LPCYSVARI	DKFZ, Heidelberg, Germany
JCV-VP1-p83-91, VARIPLPNL	DKFZ, Heidelberg, Germany
JCV-VP1-p113-121, IGVTSLMNV	DKFZ, Heidelberg, Germany

JCV-VP1-p244-p252, GPLCKGDNL	DKFZ, Heidelberg, Germany
JCV-VP1-p290-298, NPYPISFLL	DKFZ, Heidelberg, Germany
JCV-VP1-p306-314, TPRVDGQPM	DKFZ, Heidelberg, Germany
JCV-VP1-peptide pool (OP1 to OP86) *	peptides&elephants GmbH, Postdam
JCV-VP1 overlapping peptides (OP) *	peptides&elephants GmbH, Postdam
JCV-VP1-VLP	Dept. of Eukaryote Genetic Engineering, Institute of Biotechnology, Vilnius University
BKV-VP1-VLP	Dept. of Eukaryote Genetic Engineering, Institute of Biotechnology, Vilnius University
CMVpp65-p495-503, NLVPMVATV	GL Biochem (Shanghai) Ltd.; Minhang Shanghai
CMVpp65-p492-506, LARNLVPMVATVQGQ	GL Biochem (Shanghai) Ltd.; Minhang Shanghai
CMVpp65-p417-426, TPRVTGGGAM	peptides&elephants GmbH, Postdam
IMP-p58-66, GILGFVFTL	GL Biochem (Shanghai) Ltd.; Minhang Shanghai

* Detail in supplementary data

2.1.9 Healthy donors (HDs)

Buffy coat samples of healthy donors were obtained from Universität Rostock, Blood Bank and from the Institut für Klinische Transfusionsmedizin und Zelltherapie (IKTZ), Heidelberg, Blood Bank. Informed consent was obtained from all donors. PBMCs were separated using Ficoll-hypaque solution (Biochrom, Berlin, Germany). Briefly, buffy coat samples were diluted in phosphate-buffered saline (PBS) (Invitrogen, Darmstadt, Germany) layered on the dense Ficoll-hypaque separating solution and centrifuged at 2,000 revolutions per minute (rpm) for 20 minutes at room temperature (RT) without brake. This density gradient centrifugation separated leukocytes from other cells forming an interface. Carefully aspirated interface layer were collected in a new tube, washed three times with PBS and cryopreserved in 90% heat-inactivated fetal bovine serum (FBS) and 10% Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Steinheim, Germany) solution according to the standard protocol. Cryo-preserved PBMCs were stored in liquid nitrogen until used.

2.1.10 Patient sample

A patient who had a severe HC almost 10 years back was admitted in University Clinic, Heidelberg, due to chronic GvHD of the lung. The patient was treated by extracorporeal photopheresis (ECP). After obtaining the patients informed consent, the blood sample was drawn and processed for the isolation of PBMCs and cryo-preserved in liquid nitrogen until used.

2.1.11 Cell lines

Stably transfected K562 cell lines for HLA-A1, HLA-A2, HLA-B7 and HLA-B8 were a kind gift from Prof. Thomas Woelfel (University of Mainz, Germany). K562 wild type (non-transfected) and K562 expressing HLA-A2 allele were also obtained from Prof. Stefan Stevanovic (University of Tuebingen, Germany).

All the cell lines were checked for mycoplasma contamination by PCR before using in any experimental assays.

K562-non-transfected (K562 wild type)	From Prof. Stefan Stevanovic (University of Tuebingen, Germany)
K562-non-transfected (K562 wild type)	From Prof. Thomas Woelfel (University of Mainz, Germany)
K562-non-transfected (K562 wild type)	From Prof. Peter Terness (University of Heidelberg, Germany)
K562-A1 (HLA-A1-transfected K562 cells)	From Prof. Thomas Woelfel (University of Mainz, Germany)
K562-A2 (HLA-A2-transfected K562 cells)	From Prof. Thomas Woelfel (University of Mainz, Germany)
K562-A2 (HLA-A2-transfected K562 cells)	From Prof. Stefan Stevanovic (University of Tuebingen, Germany)
K562-B7 (HLA-B7-transfected K562 cells)	From Prof. Thomas Woelfel (University of Mainz, Germany)
K562-B8 (HLA-B8-transfected K562 cells)	From Prof. Thomas Woelfel (University of Mainz, Germany)
T2 cells	From Dr. med. Michael Hundemer (University of Heidelberg, Germany)

2.2 METHODS

2.2.1 Human leukocyte antigen (HLA)-typing of HDs

Low resolution two digits HLA-typing was done for all the HDs. Briefly PBMCs were thawed, counted and either stained with anti-HLA antibodies or processed for PCR.

2.2.1.1 Typing by antibodies

Thawed PBMCs were washed, counted and cell pellets were re-suspended in 100 µl of FACS buffer (containing PBS with 0.5% BSA and 0.5M EDTA) and stained with anti-HLA-A2 (BB7.2, Biolegend, San Diego, CA, USA), HLA-A1, HLA-B7 and HLA-B8 antibodies with appropriate isotype controls. Antibody staining was performed at 4°C for 20 minutes in dark. Cells were washed once and then re-suspended in FACS buffer followed by flow cytometric analysis.

2.2.1.2 Typing by single specific primer-PCR (SSP-PCR)

a) <u>Isolation of DNA from PBMCs:</u>

Thawed PBMCs were counted and washed once with PBS. The pellet was re-suspended with a lysis solution containing 4.5 ml of lysis buffer II, 125 µl of 10% SDS and 1,100 µl of 5M sodium perchlorate. After 30 seconds of incubation at RT with vigorous shaking, 2 ml of 6M NaCl was added to precipitate protein. After another 30 seconds of incubation at RT, suspension was centrifuged twiced for 5 minutes at 1,500 g to pellet all the precipitated proteins. Every time clear supernatant was taken in new falcon tube. After centrifugation, 8 ml of isopropanol was added to precipitate DNA. Precipitated DNA was carefully taken out using glass pipette, washed twiced with 70% ethanol and dissolved in 200 µl of sterile H_2O at 55^0C with continuous shaking. The concentration and purity of dissolved DNA was determined by the

nanodrop 2000c device. All the DNA preparations from PBMCs were more than 90% pure.

b) PCR and gel electrophoresis:

HLA-types A*02 and B*07 were determined by a PCR-based method using single specific primers (SSP) for HLA-A*02 and HLA-B*07. For each tube of PCR (25 µl), master mix containing (2.5 µl PCR buffer, 19.8 µl nuclease-free water, 0.5 µl dNTPs, 1 unit of Taq polymerase) was prepared. No DNA control tubes and test DNA tubes were prepared with 1 µl each of nuclease-free water and DNA (100 ng/µl) respectively. An appropriate primer set was added in the master mix (see primer table).

SSP-PCR products were electrophoresed in 1 x TBE buffer and 0.05% ethidium bromide containing 2% agarose gels. 100 bp molecular weight marker (ladder) was used in each row and after every 9 wells to identify positive bands. The gels were run for 45 minutes at 100 V and then visualized using UV trans-illuminator.

2.2.2 Lympho-proliferative assay

2.2.2.1 Mixed lymphocyte peptide culture (MLPC)

Thawed PBMCs were washed once with complete medium consists of Roswell Park Memorial Institute (RPMI-1640) containing 1% penicillin/streptomycin (Invitrogen Gibco, Grand Island, USA) and 1% L-Glutamine (Biochrom AG). Cells were then washed with separation buffer containing MACS buffer (AutoMACS rinsing solution, Miltenyi Biotec, Bergisch-Gladbach, Germany) and 0.5% bovine serum albumin (BSA, Miltenyi Biotec, Bergisch-Gladbach, Germany). $CD8^+$ and $CD8^-$ T cells were separated as positive and negative fraction through a MACS column (Miltenyi). $CD8^-$

cells, which served as APCs were irradiated with 30 Gy and loaded with appropriate peptides, peptide pool or proteins. $CD8^-$ cells cultured in complete medium without any peptide stimulations served as negative control. After 2 hours of peptide loading, $CD8^-$ cells were co-cultured with $CD8^+$ T cells in a ratio of 4:1 ($20x10^5$: $5x10^5$) in 24-well plates. The ratio of 4:1 for $CD8^-$ and $CD8^+$ T cells were also maintained when MLPC was done in 96-well plates. Plates were incubated overnight at 37°C, 5% CO_2 and supplemented with 2.5 ng/ml IL-2 (Sigma Aldrich, Steinheim, Germany) and 20 ng/mL IL-7 (Miltenyi Biotec, Bergisch-Gladbach, Germany). Cells were incubated further for 6 more days before harvesting for the specific experimental assays. For some assays, cells were restimulated with peptide loaded autologous PBMCs or K562 cells each week. Each well was fed with IL-2 and Il-7 every 2-3 days during this procedure.

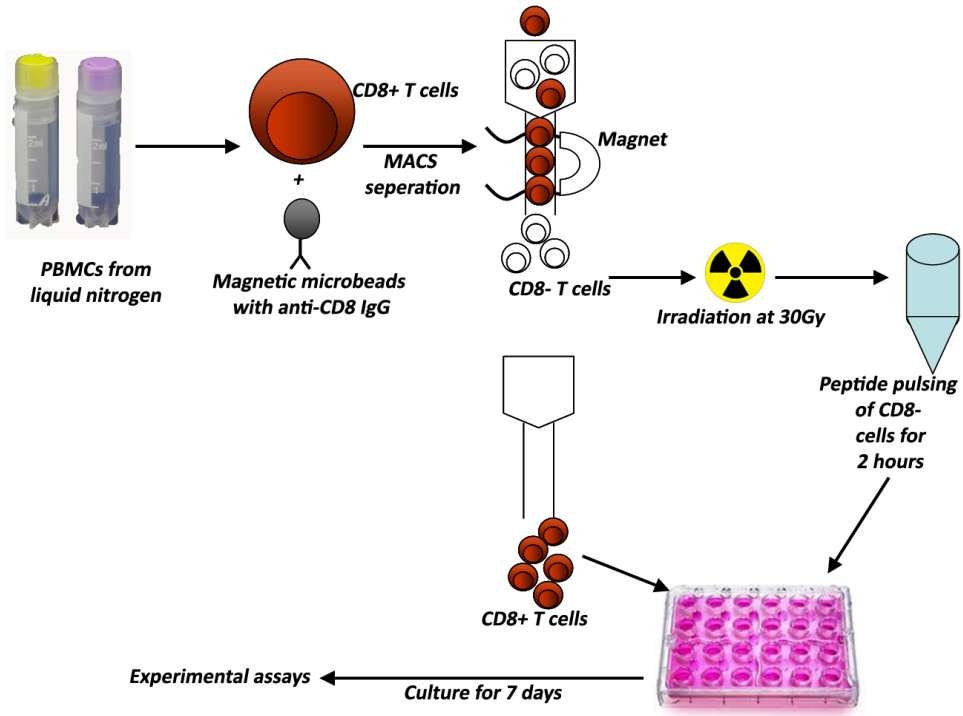


Figure 6: Scheme representing MLPC procedure.

2.2.2.2 "Whole PBMC" approach

PBMCs were thawed, washed with RPMI-1640 containing 1% penicillin/ streptomycin (Invitrogen Gibco, Grand Island, USA) and 1% L-Glutamine (Biochrom AG) and plated as $25x10^5$/ml each well in a 24-well format plate. Peptides or proteins were added directly in the wells containing PBMC. On second day and every 2-3 days, cells were supplemented with 2.5 ng/ml IL-2 (Sigma Aldrich, Steinheim, Germany) and 20 ng/mL IL-7 (Miltenyi Biotec, Bergisch-Gladbach, Germany). After a total of 7 days, cells were either harvested and used in experimental assays or restimulated with autologous PBMCs or K562 cells.

2.2.3 CFSE proliferation assay

CFSE (Invitrogen, Grand Island, NY, USA) was used as a fluorescent cell staining dye to monitor lymphocyte proliferation due to its property of progressive halving of fluorescence within daughter cells after each cell division. PBMCs were thawed and washed twice with PBS to get rid of serum proteins and then stained with 1mM of CFSE for 5 minutes at RT with continuous shaking. After 5 minutes medium containing serum was added to stop the reaction and cells were washed 3 times with complete medium before placing into culture plates. CFSE staining was measured with other fluorescent antibodies to observe the proliferation behavior of different lymphocyte populations by flow cytometry.

2.2.4 JCV-specific T cell screening

For the purpose of detecting JCV-VP1-specific T cells, HDs were screened by IFN-γ ELISPOT assay for positive signal using VP1-peptide pool. Briefly, MLPC was performed where $CD8^-$ cells were loaded with JCV-VP1-peptide pool and after 7

days of co-culture with $CD8^+$ T cells, IFN-γ ELISPOT assays were performed. Since peptide pool contains pentadecamer length peptides comprising whole VP1-protein, it was possible to screen HDs for VP1-specific T cells in a HLA-independent manner.

2.2.5 ELISPOT assay

2.2.5.1 IFN-γ secretion assay

ELISPOT plates (Multiscreen IP 96-well plates, Millipore, Massachusetts, USA) were first pre-wet activated by adding 15 µl, 35% ethanol, washed with PBS and then coated with anti–human IFN-γ (Mabtech, Nacka Strand, Sweden) re-suspended in PBS. Plates were then incubated overnight at 4^0C. Second day plates were washed with PBS to remove unbound antibodies and then blocked with PBS containing 10% FBS for 2 hours at RT. Autologous PBMCs and K562 cells loaded with peptides were used as target cells. Appropriate negative and positive control wells were prepared with complete medium and SEB-stimulation respectively. Effector cells from the cultured wells were then co-cultured with peptide loaded target cells in ELISPOT plate and incubated at 37°C, 5% CO_2 overnight. Third day plates were washed with PBS containing 0.05% tween 20 (Sigma-Aldrich) and biotin-linked secondary antibody was added in each well followed by 2 hours incubation at RT. Again after wash with PBS containing 0.05% tween 20, streptavidin-alkaline phosphatase (ALP) or streptavidin-horseradish peroxidase (HRP) was added and incubated for 2 hours at RT. Following washes were first done with PBS containing 0.05% tween 20 and once with PBS subsequently with substrate buffer. Substrate solution was prepared, added and reaction was developed for 3 minutes until visible spots were detected in positive control wells. Reaction was stopped with tap water

and plate was air dried. Visible spots were counted and analyzed using an automated ELISPOT reader (CTL, Bonn, Germany).

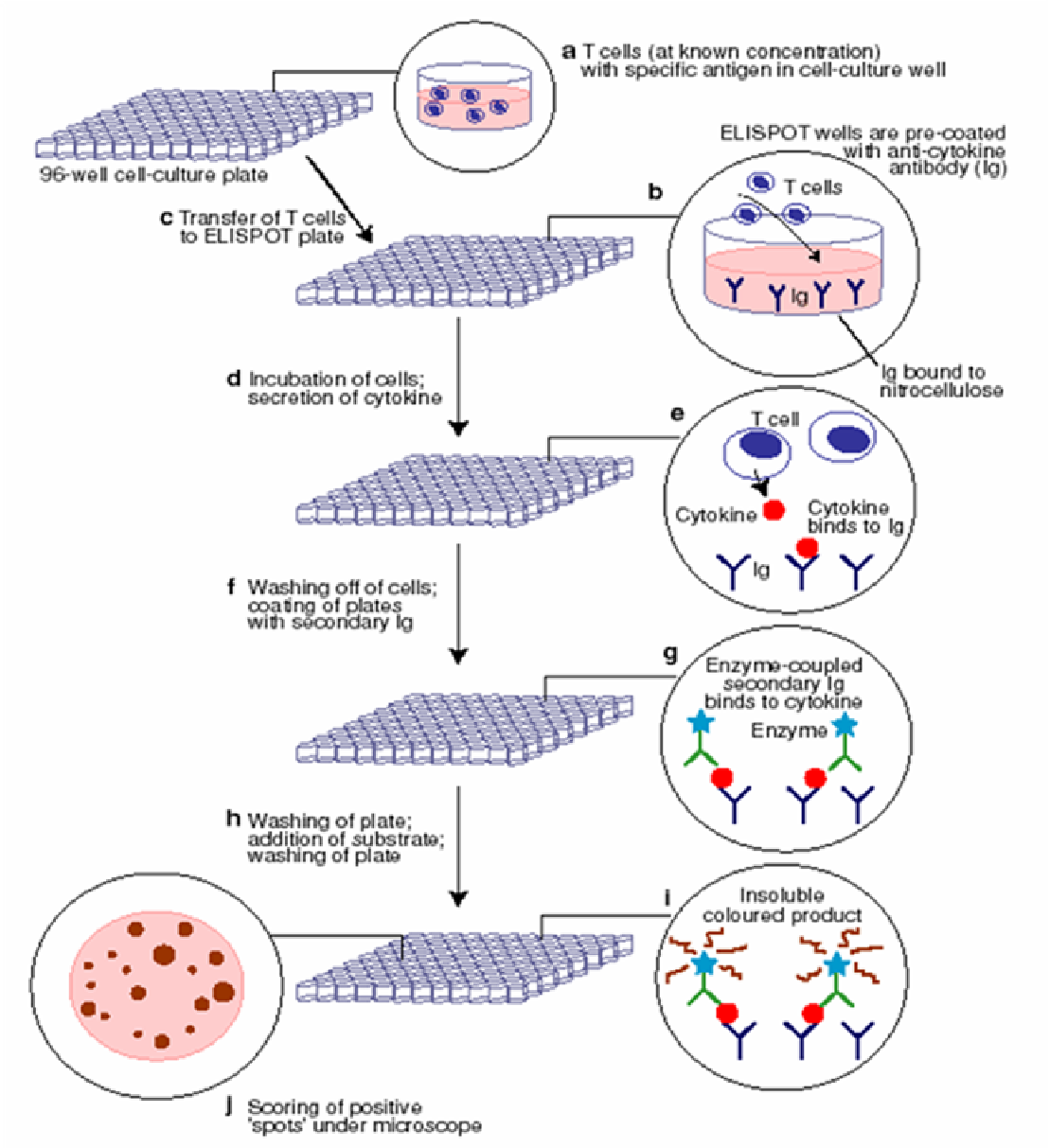


Figure 7: General scheme representing ELISPOT assay. (Hickling 1998)

2.2.5.2 Granzyme B secretion assay

Granzyme B is a serine protease, released by cytoplasmic granules of cytotoxic T cells. Granzyme B along with perforin induces cell death in virus-infected cells.

Granzyme B secretions assays were performed as described for the IFN-γ ELISPOT apart from use of Granzyme B-specific capture and secondary biotinylated antibody.

2.2.6 Flow cytometry

2.2.6.1 Surface marker staining

All the surface marker antibodies were first titrated and optimum concentration was used for flow cytometric staining. Briefly, cells were washed with FACS buffer, stained for appropriate antibodies and incubated for 20-30 minutes at 4^0C. In case of TCR-specific MHC-tetramers, stained cells were incubated for 45 minutes at RT followed by the surface marker staining for 20-30 minutes at 4^0C. After staining, cells were washed with FACS buffer and re-suspended in the same buffer for flow cytometric analysis. A minimum of 50,000 events were acquired during acquisition and data were further analyzed using flow cytometry analysis software (FlowJo®, Tree Star, Inc, USA) and Facs Diva (FACSDiva, BD biosciences).

2.2.6.2 IFN-γ intracellular staining

Intracellular staining for IFN-γ was done according to the described protocol by Jung et al. (Jung, Schauer et al. 1993). Briefly, cells were restimulated overnight with peptides, peptide pool and proteins. After 2 hours, 10 µg/ml brefeldin-A (BFA) was also added to each well. Cells were washed with cold FACS buffer and then stained for the surface antigens. Cells were washed twice with FACS buffer and fixed using 500 µl, 4% paraformaldehyde at RT for 5 minutes. After washing once with permeabilization buffer (FACS buffer with 0.1% saponin, Sigma), cells were re-suspended in permeabilization buffer and incubated at 4^0C for 30 minutes. After washing once with permeabilization buffer, staining was done for 30 minutes at 4^0C

with anti- IFN-γ antibody and isotype control prepared in the same buffer. Cells were once washed, re-suspended in FACS buffer and at least 50,000 events were acquired during acquisition. Data were further analyzed as previously described.

3 RESULTS

3.1 Characterization of the relative frequency of polyomavirus-specific T cells in the peripheral blood

In the peripheral blood of HDs and patients, precursor frequency is described as the number of T cells specific for any given antigen. Precursor frequency may vary a lot depending on the type of antigens (van Aalderen, Remmerswaal et al. 2013). Recent technical advances like MHC-tetramers and cytokine measurement after stimulation with synthetic peptides have allowed accurate quantification of the number of $CD8^+$ T cells. To augment the T cell response and to increase the read-out sensitivity, protocols for T cell expansion are being used. Though, *in vitro* expanded T cells do not represent the real precursor frequencies, they do reflect the relative numbers of T cells.

Here, relative T cell frequencies for polyomaviruses were compared with influenza virus and CMV-specific T cells. The latter have been extensively described in the literature (Borysiewicz, Graham et al. 1988; Lee, Oelke et al. 2011). Known immunogenic peptide sequences for JCV, CMV and influenza virus were used to stimulate PBMCs in an expansion protocol. Specific MHC-tetramers were used to detect $CD8^+$ T cells in flow cytometric assays.

We found that the frequencies of polyomavirus-specific T cells were significantly lower than that of CMV and influenza virus. Out of eighteen, fifteen HDs (83%) showed low frequencies (≤ 0.5%). Noteworthy that at least one donor showed a sufficient number (>2%) of $CD8^+$ T cells specific to the JCV-p100-peptide (Table 2).

One recently published study showed similar results using immunodominant BKV-VP1-p108-peptide (van Aalderen, Remmerswaal et al. 2013). Compared to p100, even lower numbers of JCV-VP1-p36-specific T cells were measured. In seven HDs tested for p36-specific T cells, we found four with no response and rest of the three with low percentage of specific T cells (≤0.2) (Table 2).

Table 2: Relative frequency of JCV, CMV and influenza virus-specific $CD8^+$ T cells. Numbers represent the percentage of MHC-tetramer and CD8 double positive T cells. ID represents the last three to four digits from the blood bank code for the healthy donors (HDs).

ID	JCV-VP1 (p36-44)	JCV-VP1 (p100-108)	CMVpp65 (p495-503)	CMVpp65 (p417-426)	Influenza matrix protein (p58-66)
4148	-	0.07	-	-	4.63
4318	-	0.0	-	6.62	-
4040	-	0.0	-	51.9	-
4179	-	0.0	-	-	5.14
895	0.0	0.5	2.1	-	-
5143	0.0	0.0	10	-	-
110	0.1	0.2	-	-	-
114	0.2	0.1	-	-	-
207	0.2	0.4	0.1	-	-
425	0.0	0.1	-	-	-
797	0.0	0.1	-	-	-
4410	-	0.2	1.5	-	-
638	-	0.5	53.6	-	-
211	-	1.27	1.37	-	-
711	-	5.4	7.5	-	-
872	-	2.05	2.44	-	-
713	-	0.0	2.55	-	-
771	-	0.56	53.1	-	-

"-" indicates not tested

On the other hand, CMV and influenza virus peptide-specific T cells were detectable in most of the patients. Using two immunodominant peptides derived from CMVpp65 restricted to HLA-A2 and HLA-B7, we found that out of twelve, only one donor showed <1% of MHC-tetramer positive T cells (Table 2). In three donors, an exceptionally high T cell response was measured where more than half of the $CD8^+$

T cells were specific to the CMVpp65-derived peptide (Table 2). Similarly, we also found high T cells for IMP-derived peptide. Although, in this study only four donors were tested for CMVpp65-peptide (p417-426) and for the IMP-derived peptide, results showed that all the donors tested had high relative frequencies of peptide-specific T cells (Table 2). Based on these results, it is clear that the number of circulating polyomavirus-specific T cells in HDs is very low when compared to other viruses (e.g. CMV and influenza virus).

3.2 Strategies

3.2.1 MLPC versus "whole PBMCs" approach

As discussed in the previous section, $CD8^+$ T cells specific to polyomaviruses are in general very low. To measure this low-frequency of anti-viral T cells, sensitive expansion protocols are needed. We compared two protocols using immunodominant CMVpp65-peptide (p495-503) in CMV seropositive HDs. First, we performed MLPC where autologous $CD8^+$ T lymphocytes and $CD8^-$ fraction of PBMCs served as responder and stimulator cells, respectively. Secondly, we performed "whole PBMCs" approach, where PBMCs were directly loaded with peptides without prior separation. This latter approach was compared to MLPC for several reasons. It is easy to perform, inexpensive and also time-saving, whereas MLPC technique requires expensive tools like microbeads, magnets, columns and filters. After a week of culture, CMVpp65-peptide-specific $CD8^+$ T cells were visualized by flow cytometry using MHC-tetramers and antibodies directed to CD8 and CD137. CD137 was used as a marker for functional-activated T cells which has shown to be up-regulated uniformly on all responding cells after 24 hours of stimulation (Wolfl, Kuball et al. 2007).

There was significant T cell stimulations directed to the CMVpp65-peptide using both expansion protocols. However, the overall $CD8^+$ T cell responses were clearly higher in MLPC as visualized by MHC-tetramer positive $CD8^+$ T cells (Figure 8 A). Additionally, relative percentage of $CD8^+$ T cells were also seen to be higher in MLPC compare to "whole PBMCs". It was probably because in MLPC $CD8^-$ T cells were irradiated therefore did not contributed in the overall proliferation of cells which eventually elevated the relative number of $CD8^+$ T cells.

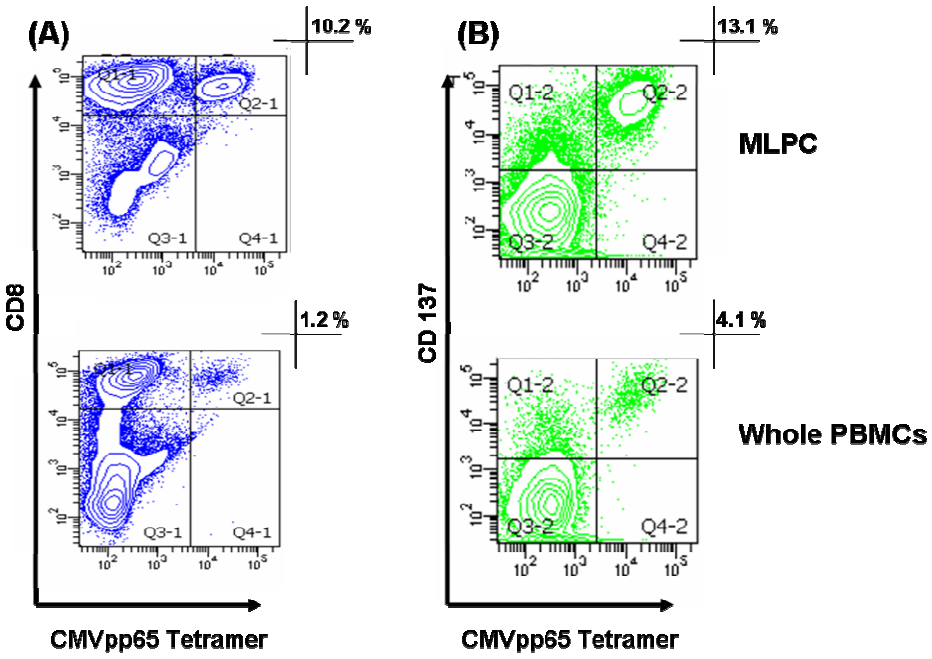


Figure 8: Comparison of MLPC versus "whole PBMCs stimulation". Phenotype and function of T cells were analyzed after MLPC and "whole PBMCs approach" respectively. The experimental read-out was performed by multicolor flow cytometry. (A) CD8 versus peptide-specific MHC-tetramer immunofluorescence: Cells were gated on lymphocytes. (B) CD137 versus peptide-specific MHC-tetramer immunofluorescence: Cells were gated on $CD8^+$ T cells.

Using CD137, we also observed that all the tetramer positive $CD8^+$ T cells were also CD137 positive, which indicates the functional activity of T cells (Figure 8 B).

We also compared both culture approaches using other viral antigens and found similar results (data not shown). Overall, for expanding very low frequencies of anti-viral T cells in an efficient manner, MLPC was shown to be superior compared to "whole PBMCs" protocol.

3.2.2 Optimization of T cell stimulation

3.2.2.1 Antigen presenting cells (APCs)

Activation of CTLs requires APCs of certain qualities. First, APCs should not lead to a background of non-specific T cell stimulation and secondly, APCs should possess a good antigen presenting capability. Additionally, the ratio of APCs to effectors cells constitutes a crucial factor which has a direct impact to trigger T cells.

Here we tried to optimize and standardize our read-out for an efficient T cell response using different APCs at various ratios to effector T cells. We compared different types of APCs like autologous $CD8^-$ T cells, PBMCs, mDCs, T2 cells and transfected lines of K562. K562 cells transfected with HLA class I alleles were used as APCs in restriction analysis. T cell responses were initiated using the CMVpp65-derived peptide (p495-503) in a MLPC. The outcome of the stimulation was measured by ELISPOT assay and multicolor flow cytometry.

We observed that autologous cells as well as cell lines did stimulate T cell responses in a ratio dependent manner. Autologous PBMCs at a ratio to effector cells of 10:1 were able to stimulate significant T cell responses with low background (Figure 9). Moreover, it was seen that at higher ratios than this, T cell responses were not improving, and the background was also very high (data not shown). Using K562-A2 and T2 cell lines we found that APCs to effector cells ratio of 1:1 is best and

triggered a T cell stimulation similar to autologous PBMCs. Additionally, it was observed that the negative fraction of $CD8^+$ T cells was not as good as other APCs in restimulating T cells. This could be probably the case as these cells went through freeze-thaw cycles ≥2 times which made them comparatively less capable in presenting peptides to effector cells. For future experiments, $CD8^-$ cells were not considered anymore for restimulation in ELISPOT assays.

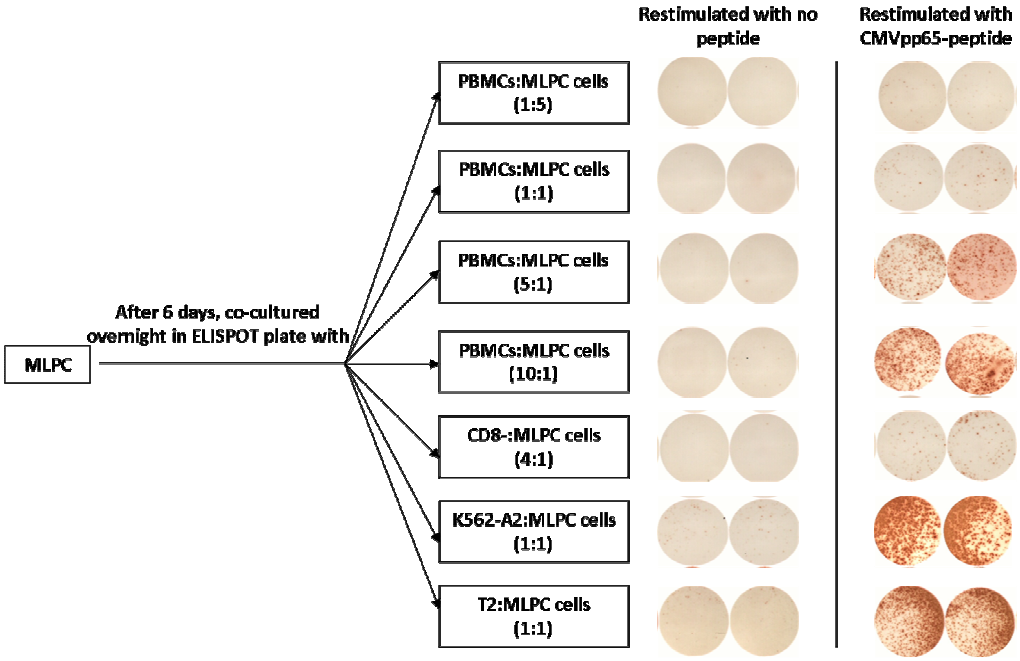


Figure 9: Comparison of APCs: Representation of an IFN-γ ELISPOT assay after MLPC using different ratios of autologous PBMCs and $CD8^-$ cells, as well as cell lines T2 and HLA-A2-transfected K562 cell lines.

Consequently, autologous PBMCs and cell lines both were shown to be equally effective in initiating significant T cell response at certain ratios to effector T cells.

K562 cells as APCs:

Furthermore, for the analysis of HLA restriction in an *in vitro* assay, non-transfected and transfected K562 cells were used. K562 cells transfected with HLA-A2 allele has

been previously shown to be efficient in stimulating T cells in ELISPOT assays (Britten, Meyer et al. 2002).

We started with the comparison of K562 wild type (non-transfected) and transfected cells from two or more sources. Before utilizing the cells in an *in vitro* assay, K562 cells and transfectants were analyzed for the absence of HLA class I expression (Figure 10 A) and the presence of HLA-A2 (Figure 10 B) allele by flow cytometry. To perform restriction analysis, we compared autologous PBMCs with K562 non-transfected and transfected cell lines in ELISPOT assays using HLA-A2 restricted peptide derived from CMVpp65. Here, K562-A2 as well as autologous PBMCs served as positive controls. K562 wild type and K562-B8 cells were used as negative controls. We found that the CMVpp65-derived peptide which does not bind promiscuously across HLA class I molecules, was presented by K562-A2 and autologous PBMCs but not by K562 wild type and K562-B8 cells (Figure 10 C). It is clear that K562 wild type cells due to lack of HLA class I molecule and K562-B8 due to incompatible HLA class I molecule, were unable to bind and present the HLA-A2 restricted peptide.

To understand whether K562 cells can efficiently stimulate T cell responses in the absence of autologous APCs, we stimulated freshly isolated $CD8^+$ T cells with K562-A2 and non-transfected K562 cells. It was observed that there was significant T cell response stimulated by K562-A2 cells as seen by IFN-γ secretion in ELISPOT assay (Figure 10 D).

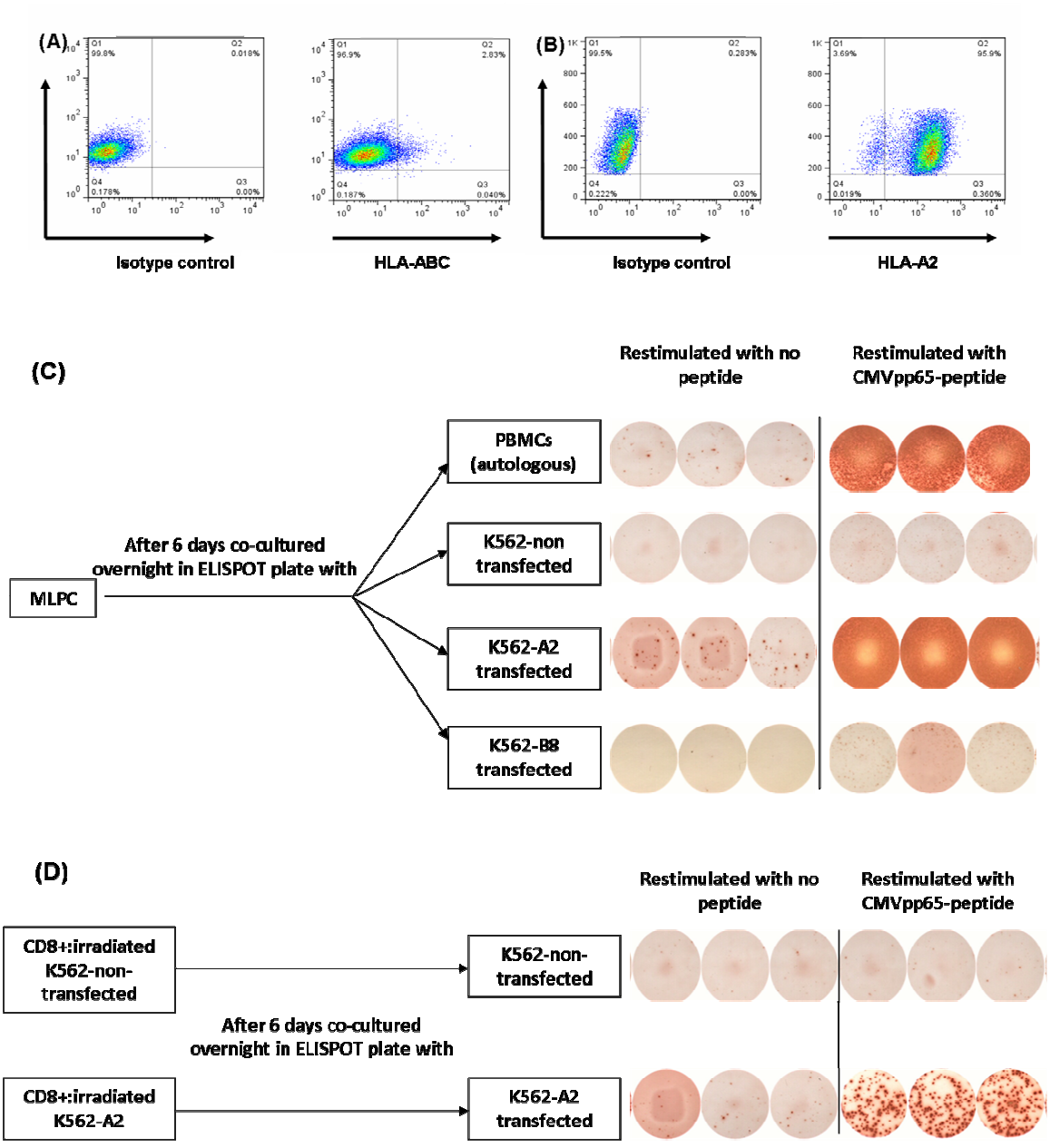


Figure 10: Antigen presentation properties of K562 wild type (non-transfected) and transfected cells: (A) Flow cytometric measurement of HLA class I expression on K562 wild type cells versus. (B) HLA-A2-transfected K562 cells. (C) Representation of an IFN-γ ELISPOT assay using autologous PBMCs, K562 and transfected lines for HLA-A2 and HLA-B8. (D) MLPC followed by an IFN-γ ELISPOT assay using K562 wild type versus HLA-A2-transfected K562 cells.

In conclusion, K562-transfected cells were very suitable tools not only to utilize them efficiently as APCs but also to study HLA restriction in an *in vitro* culture system.

3.2.2.2 Peptide length and concentration

Optimal length of peptide is also one of the crucial factors deciding the magnitude of specific T cell response. Short peptides (9-11 amino acids) as well as long peptides (15-40 amino acids) can induce $CD8^+$ T cell responses. Short peptides can bind directly and can be presented without the need of processing by the HLA class I molecule while longer peptides need to be internalized, processed and then presented which is efficiently done by more professional APCs (e.g. DCs). Some studies showed that longer peptides are more immunogenic compared to short peptides (Zwaveling, Ferreira Mota et al. 2002; Sercarz and Maverakis 2003; Bijker, van den Eeden et al. 2008). Moreover, *in vivo* mouse tumor studies showed that the extended processing of longer peptide leads to superior induced memory $CD8^+$ T cell response than that of shorter peptides (Zwaveling, Ferreira Mota et al. 2002). However, the effect of the peptide length on the induced immune response is still under current discussion.

We compared T cell responses induced by CMVpp65-derived immunodominant 9mer (nonamer) peptide with a 15mer (pentadecamer) peptide which contains flanking residues (Figure 11 A). Using both the peptides at same concentration (100 ng/ml) a MLPC was performed. The experimental read-out was visualized by MHC-tetramers in flow cytometry. It was observed that both individual 9mer and 15mer peptides were able to stimulate a comparable number of peptide-specific $CD8^+$ T cells (Figure 11 B). 15mer peptide stimulation of $CD8^+$ T cells indicated that the peptide was processed and then presented by APCs on an HLA class I molecule. We could also reproduce similar results with the JCV-VP1-p100-peptide (discussed in 3.3).

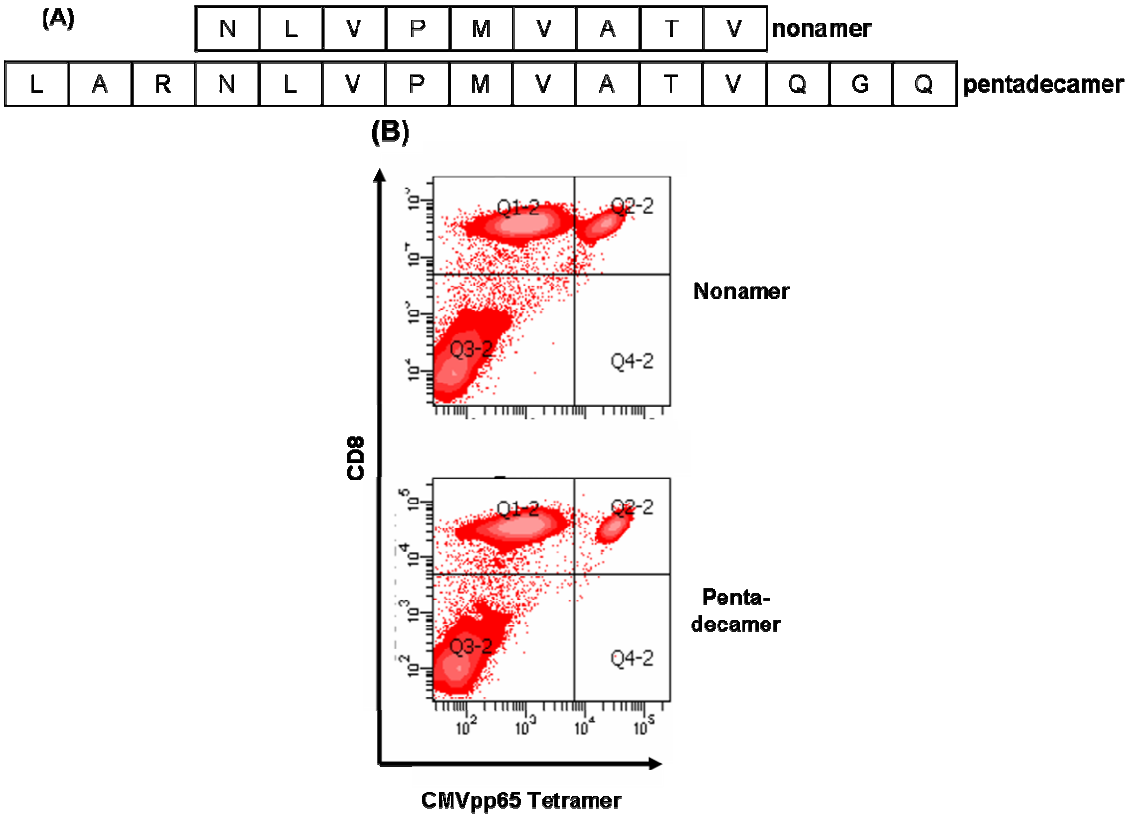


Figure 11: Comparison of 9mer versus 15mer induced T cell response. (A) An schematic alignment of the 9mer (nonamer) versus 15mer (pentadecamer) peptide derived from CMVpp65-peptide (p495-503). (B) Representative flow cytometry data after stimulation in MLPC with CMVpp65-peptide-derived 9mer versus the corresponding 15mer peptide. Cells were visualized being double-stained with peptide-specific MHC-tetramers and antibody against CD8.

To assess the importance of concentration of peptides in the setting of MLPC, we compared 9mer and 15mer in a concentration dependent manner. Both peptides were used at increasing concentrations, starting from 10 ng/ml to 10 µg/ml. We observed that both the 9mer and the 15mer peptides were able to stimulate a significant T cell response. At a low concentration (10 ng/ml), a strong T cell response was initiated using the 9mer peptide. This indicates that the 9mer peptide was efficiently presented on HLA class I molecules by APCs which eventually resulted in an excellent T cell response. Moreover, it was observed that the short

peptide gave a maximum T cell response at 10 ng/ml that could not be augmented by higher concentrations of the peptide (Figure 12).

Compared to the 9mer peptide, the T cell response induced by the 15mer peptide was also good. However, at the lower concentration (10 ng/ml) there was a dramatic decrease in the number of specific T cells. A possible explanation for the reduced T cell number might be that the 15mer peptide was not sufficiently available at this low concentration of 10 ng/ml. Keeping in mind the fact that 15mer peptides cannot directly bind to the HLA class I molecule and has to go through internalization and processing steps followed by presentation.

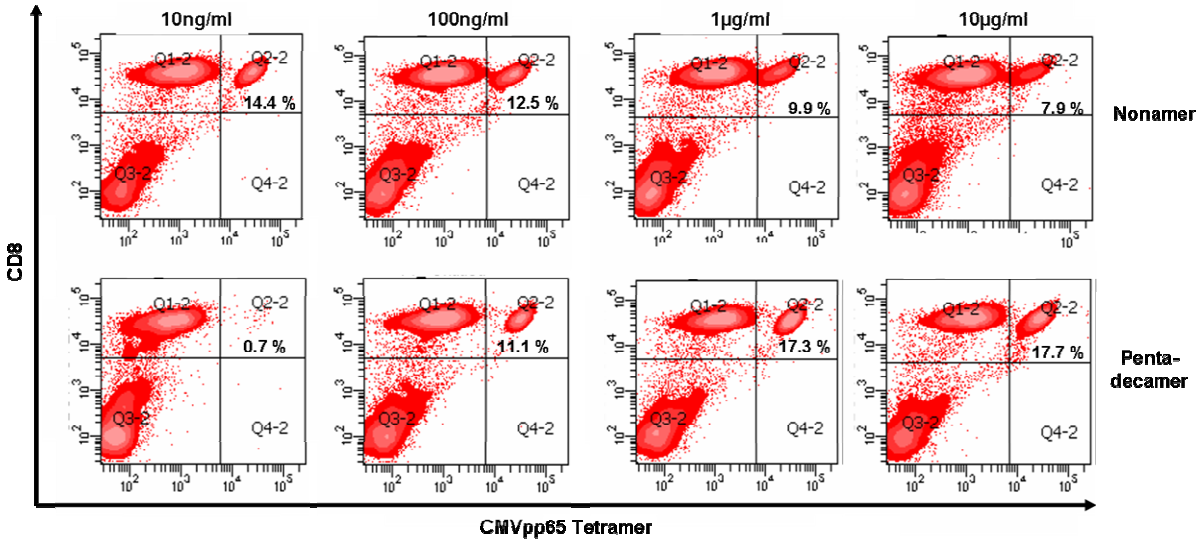


Figure 12: 9mer versus 15mer peptide, the concentration matters. Four different concentrations of CMVpp65-peptide were used: starting from left to right, 10 ng/ml, 100 ng/ml, 1 µg/ml and 10 µg/ml. Representative flow cytometry data after stimulation in MLPC with CMVpp65-peptide-derived 9mer versus the corresponding 15mer peptide. Cells were visualized being double-stained with peptide-specific MHC-tetramers and antibody against CD8.

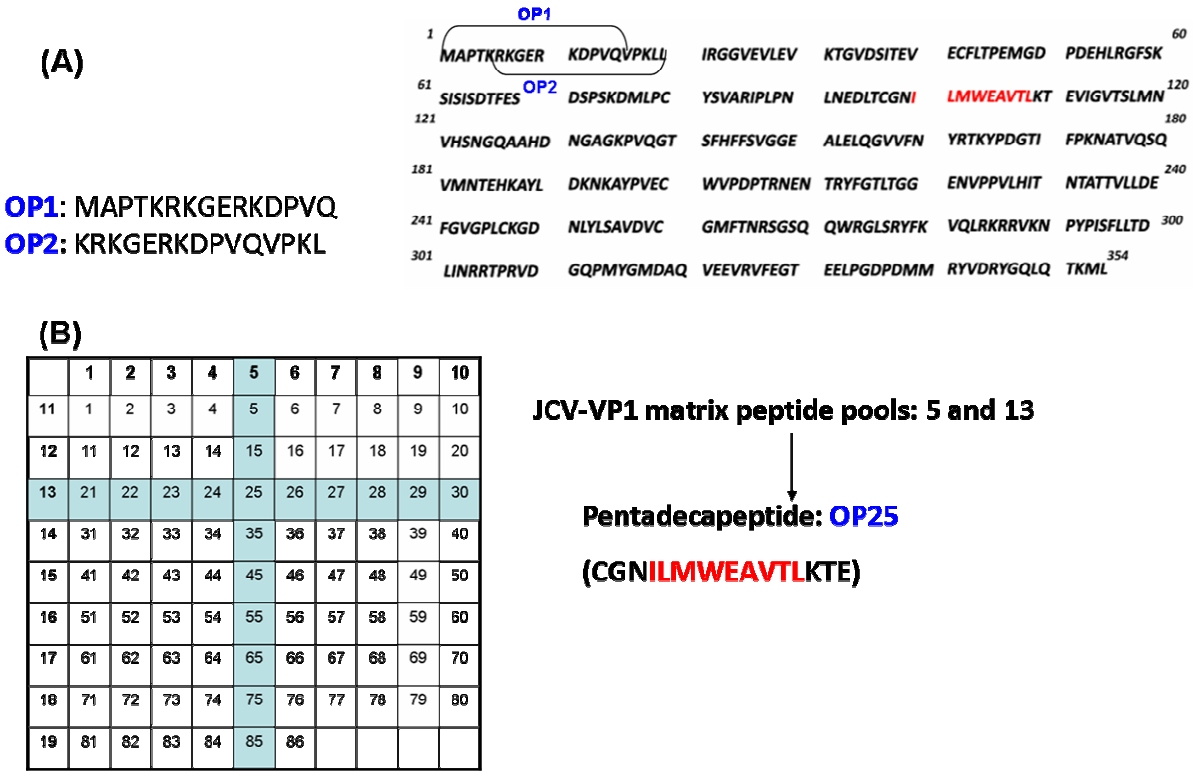
In summary, both the 9mer and the 15mer peptides are excellent in stimulating a CMVpp65-specific T cell response. Only at concentration lower than 100 ng/ml, the 9mer peptide appears to be superior.

3.2.2.3 Peptide matrix sub-pools versus single peptide stimulation

Overlapping peptides spanning the whole protein is an approach which has been used widely to identify T cell responses to more than one peptide antigen, independently from HLA restriction. Overlapping peptide sequences consisting of 15 amino acid residues with 11 amino acid residues overlap have been previously shown to stimulate both $CD4^+$ and $CD8^+$ T cell responses (Maecker, von et al. 2001).

In a comprehensive epitope mapping strategy, we used 86 overlapping peptides which covered the 354 amino acid residues of the viral protein JCV-VP1 (Figure 13 A). These overlapping peptides were arranged in a horizontal and vertical grid to make 19 peptide matrix sub-pools. Each peptide matrix sub-pool encompassed at least 6 individual overlapping peptides and due to the arrangement each individual peptide was present in 2 sub-pools (Figure 13 B). Peptide matrix pools have several advantages over individual peptides. The matrix pool approach is useful in measuring the immune response when the numbers of immune cells are limited. Therefore it reduces the amount of samples required and saves time and reagents.

To understand whether the matrix peptide pool can initiate a T cell response similarly to the 9mer peptide, we compared the T cell stimulation induced by JCV-VP1-p100-peptide (9mer) with peptide matrix sub-pools encompassing the corresponding 15mer peptide OP25. The OP25 (15mer) peptide was present in two sub-pools (numbers 5 and 13) in the matrix grid (Figure 13 B). In MLPC, the concentration of the single 9mer peptide and the matrix sub-pools was 1 µg/ml/peptide. Consequently, the total concentration of a matrix sub-pool was 10 µg/ml if it contained ten peptides. The $CD8^+$ T cell response was measured by CFSE proliferation assay and MHC-tetramer staining.



	1	2	3	4	5	6	7	8	9	10
11	1	2	3	4	5	6	7	8	9	10
12	11	12	13	14	15	16	17	18	19	20
13	21	22	23	24	25	26	27	28	29	30
14	31	32	33	34	35	36	37	38	39	40
15	41	42	43	44	45	46	47	48	49	50
16	51	52	53	54	55	56	57	58	59	60
17	61	62	63	64	65	66	67	68	69	70
18	71	72	73	74	75	76	77	78	79	80
19	81	82	83	84	85	86				

Figure 13: Overlapping peptide sequences and matrix peptide sub-pools covering the JCV-VP1 sequence: (A) General scheme of overlapping peptide sequences. Each peptide is 15 amino acid residues long and overlaps with the next peptide by 11 amino acid residues (bracket 1 and 2). (B) Instead of each peptide being tested individually, a total of 19 matrix peptide sub-pools were made from 86 JCV-VP1 overlapping peptides. Thus, each individual peptide is present in two different sub-pools. For example, when peptide sub-pools 5 and 13 are both positive (highlighted), all individual peptides present in these two peptide sub-pools could be antigenic, however the intersection of both stimulatory sub-pools indicates peptide number 25 (OP25) as antigenic peptide.

There was a significant positive signal with both the single peptide and the matrix sub-pools stimulation. However, the percentage of expanded $CD8^+$ T cells after stimulation with the single peptide (Figure 14 A) was marginally higher than those stimulated with the matrix sub-pools (Figure 14 B, C). This could be due to competition among peptides in the matrix sub-pools for processing and presentation. The concentration of the individual peptide in the matrix sub-pools and the single 9mer peptide was identical, but the molarity was slightly higher for the 9mer peptide. The difference in molarity could be another reason for the slightly higher $CD8^+$ T cell

response for 9mer peptide. Furthermore, it was seen that irrespective to single peptide or matrix sub-pools stimulation, MHC-tetramer positive $CD8^+$ T cells were all CFSE low (Figure 14 A, B, C), indicating that all tetramer positive cells have undergone proliferation.

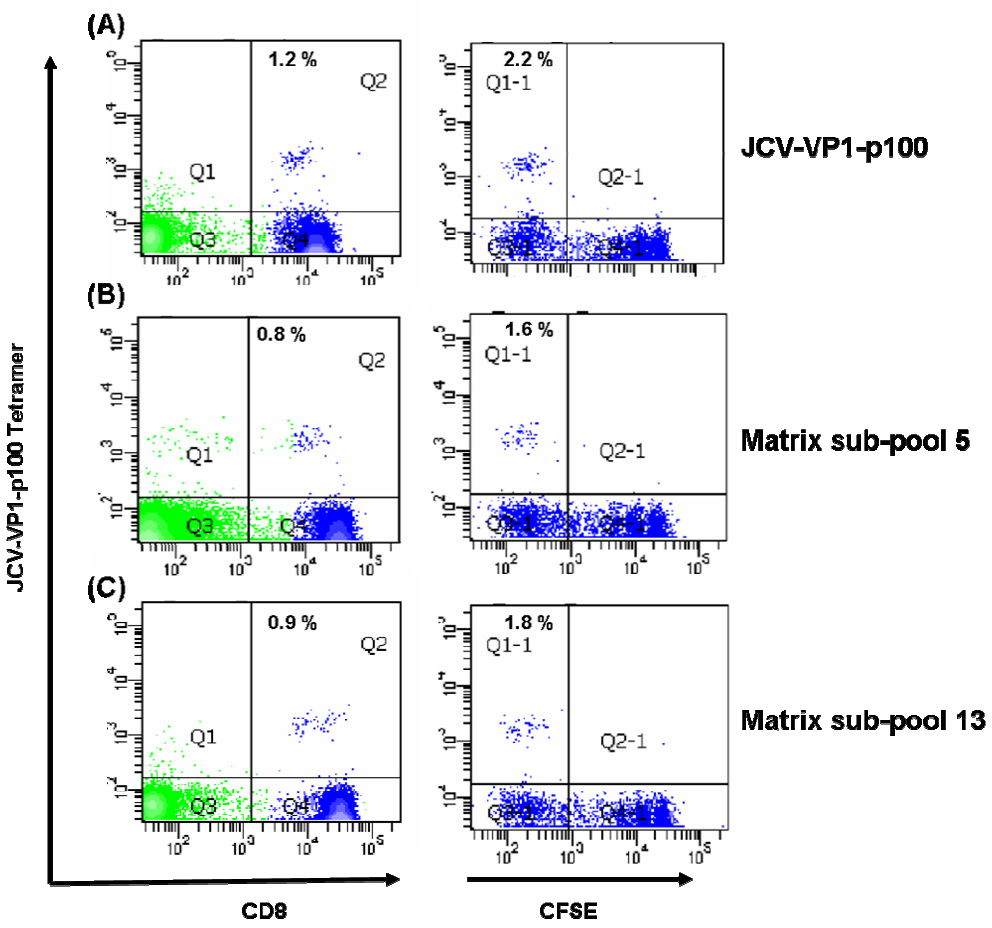


Figure 14: Comparison of T cell stimulation by single peptide versus matrix sub-pools: CFSE proliferation assay and double-staining with CD8 and MHC-tetramer was performed to visualize the JCV-VP1-p100-peptide stimulation. CD8 versus MHC-tetramer analysis dotplots were gated on lymphocytes. CFSE versus MHC-tetramer analysis dotplots were gated on $CD8^+$ T cells. MLPC assays were performed with (A) JCV-VP1-p100 9mer peptide. (B) JCV-VP1 matrix sub-pool number 5, which encompassed eight individual 15mer peptides. (C) JCV-VP1 matrix sub-pool number 13, which encompassed ten individual 15mer peptides. The OP25, a 15mer peptide was present in both sub-pools number 5 and number 13.

Overall, we found that a pool of 10 peptides does stimulate a T cell response similarly to that elicited by a single immunodominant 9mer peptide. Therefore, it constitutes an excellent tool for high throughput screening (HTS) and saves material, particularly when the sample cell numbers are limited.

3.3 Characterization of high-affinity JCV-p100-peptide-specific CD8$^+$ T cells

Out of many peptides derived from a particular foreign antigen, only few peptides can induce a measurable T cell response along with a given HLA class I molecule, a phenomenon called as immunodominance. In HLA-A2 restricted HDs and patients, p100 and p36 are two immunodominant peptides derived from the JCV capsid protein VP1 (see Table 1). The crucial factor determining the immunodominance of a given epitope is the binding affinity of the peptide to the HLA class I molecules and more importantly the affinity of TCR to the peptide-HLA (p-HLA) complex.

In a HD, who had a reasonable T cell response to the JCV-VP1-p100-peptide, we characterized specific T cells using the p100-peptide in a dose-dependent manner (10 ng/ml to 10 µg/ml). The p495-503 derived from the CMVpp65 antigen was used in parallel as a comparator peptide (Figure 15 A, B).

Performing a titration of the CMVpp65-derived peptide we observed that T cells were responding to peptide concentrations titrated down to 10 ng/ml. Looking closely, a strong T cell response was measured at the lowest concentration of 10 ng/ml (discussed in 3.2.2.2) (Figure 15 A). Using the JCV-p100-peptide, we observed an excellent T cell response at the concentration of 100 ng/ml. Surprisingly

concentration as low as 10 ng/ml also stimulated a significantly high T cell response (Figure 15 B).

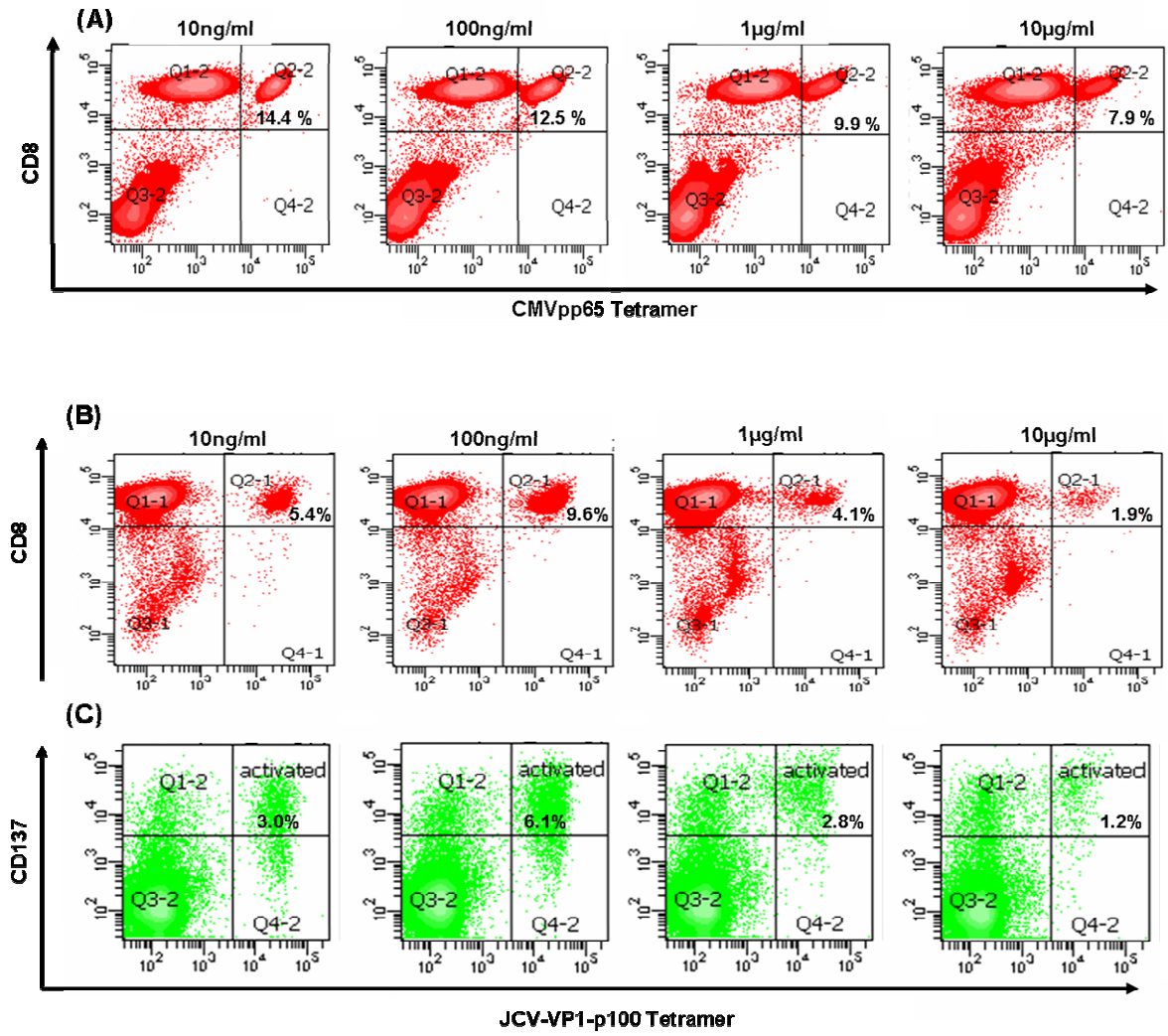


Figure 15: Detection of high-affinity T cells: $CD8^+$ T cells were stimulated using four different concentrations of peptides starting from left 10 ng/ml, 100 ng/ml, 1 µg/ml and 10 µg/ml. The experimental outcome was measured by MHC-tetramer along with CD137 and CD8 staining. (A) CMVpp65-peptide-specific MHC-tetramer versus CD8 staining. (B) JCV-VP1-p100-peptide-specific MHC-tetramer versus CD8 staining (C) JCV-VP1-p100-peptide-specific MHC-tetramer versus CD137 staining.

This high T cell response at a concentration as low as 10 ng/ml suggests that the relatively low density of p-HLA complex can efficiently interact with the TCR to elicit a significant T cell response, which is characteristic for a high-affinity TCR. In our

experiment, it was observed that the relative affinity of T cells specific to JCV-VP1 and CMVpp65 was very high (Figure 15).

Additionally, the functional reactivity was measured for the peptide-specific T cells. It was demonstrated that all T cells specific to the p100-peptide were also functionally active as seen by positive staining for CD137 (Figure 15 C). Overall, our titration experiment suggested that the T cells specific to the VP1-p100-peptide expressed high-affinity TCR and showed high functional reactivity.

Long-term culture maintenance of high-affinity T cells specific to JCV-VP1-p100-peptide

In the previous section, we observed high and low numbers of T cells at peptide concentrations of 100 ng/ml and 10 µg/ml respectively. Here, these T cells were maintained with weekly stimulation using irradiated p100-peptide loaded on K562-A2 cells. After each stimulation T cells were assessed for peptide-specificity by MHC-tetramer and for functional reactivity by anti-CD137 staining.

It was observed that p100-peptide-specific T cells stimulated with two different concentrations were maintained successfully for a period of almost four weeks. Using a peptide concentration of 10 µg/ml, a good frequency of specific T cells was observed till the 3rd week. But after the fourth consecutive stimulation there were almost no detectable T cells left (Figure 16 A). Overstimulation of T cells with high concentrations of peptides might be one possible explanation for the disappearance of T cells during long-term culture. On the contrary, when T cells were stimulated with peptides at a 100 fold dilution (100 ng/ml), significant T cell responses were observed which were maintained throughout the period of culture (Figure 16 B).

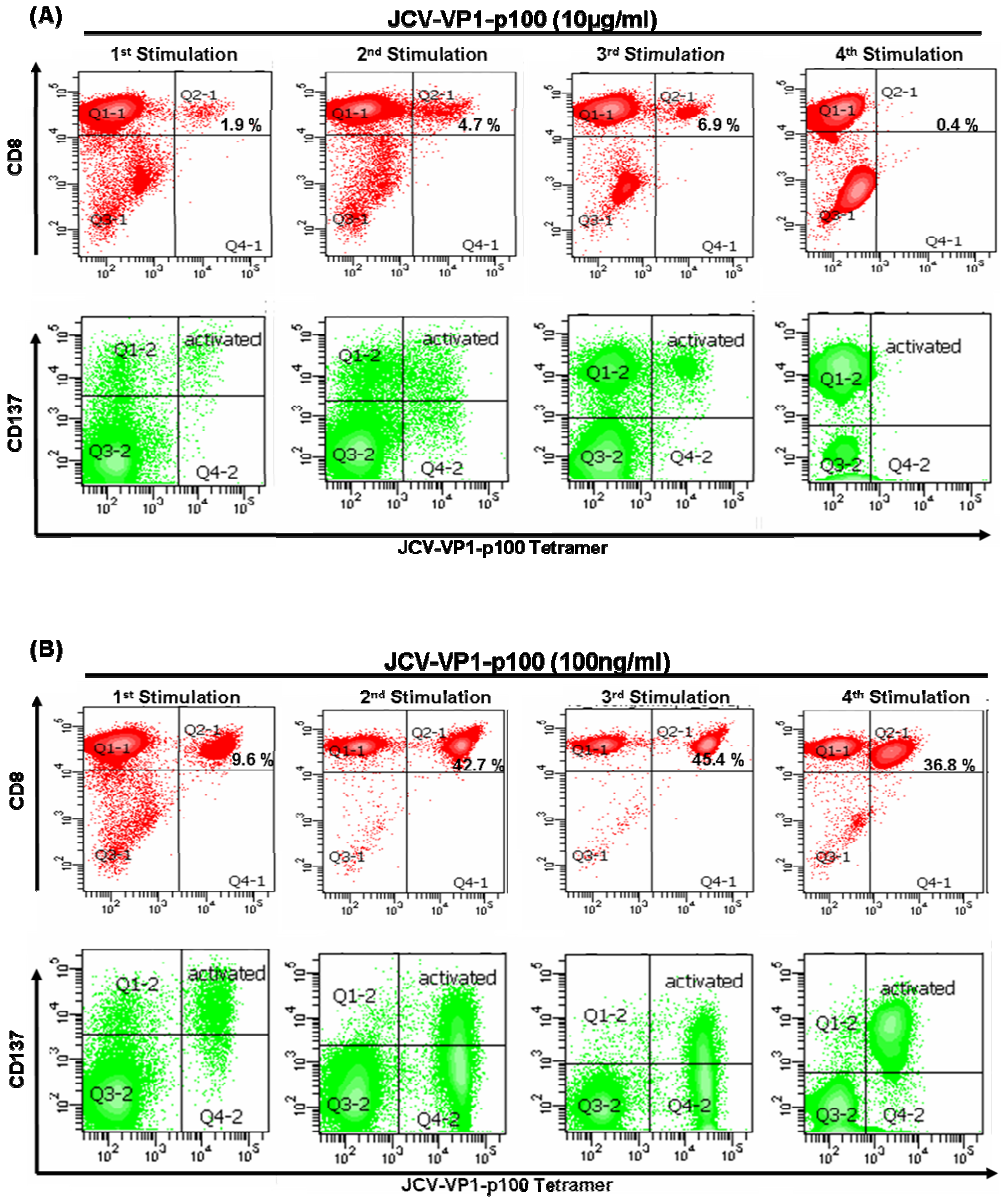


Figure 16: Long-term culture of JCV-VP1-p100-peptide-specific CD8$^+$ T cells: CD8$^+$ T cells were maintained for over four weeks with two different concentrations of JCV-VP1-p100-peptide. Cells were stained for JCV-VP1-p100-peptide-specific MHC-tetramer along with CD137 and CD8 antibodies. CD8$^+$ T cells were stimulated weekly with irradiated K562-A2 cells pulsed with (A) 10 µg/ml peptide (B) 100 ng/ml peptide.

Moreover, we observed that all T cells were functionally reactive as measured by staining for CD137. However, using peptide at a concentration of 10 µg/ml, some non-specific T cells (MHC-tetramer negative) were also detected to be CD137 positive (Figure 16 A). This was not the case when 100 ng/ml peptide was used

since all CD137 positive cells were only double-positive for peptide-specific T cells (Figure 16 B).

Overall, we were able to generate high-affinity JCV-specific $CD8^+$ T cells that were functionally active and could be maintained in an *in vitro* culture for at least four weeks.

3.4 Cross-reactivity of $CD8^+$ T cells specific to JCV and BKV

Between JCV and BKV, almost 70% of the genome and 87% of the VP1-protein sequence is homologous (Figure 17). Thus it is not very surprising that T cells are cross-reactive for these two virus-derived peptide epitopes.

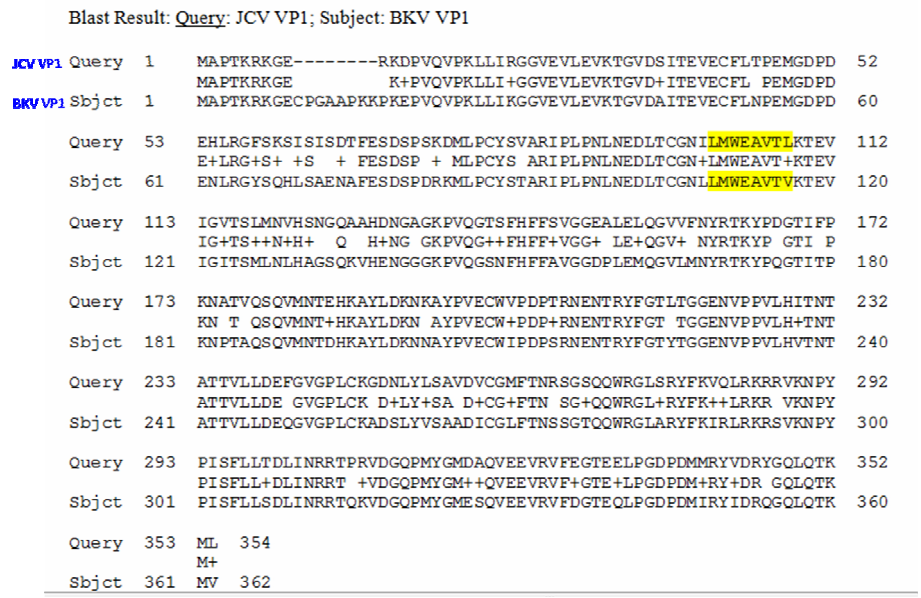


Figure 17: Alignment of JCV-VP1 versus BKV-VP1 using the data base protein BLAST (blast.ncbi.nlm.nih.gov): Query and subject represents JCV-VP1 and BKV-VP1 amino acid sequences. Highlighted are the homologous amino acid sequences for JCV-VP1-p100 (ILMWEAVTL) and BKV-VP1-p108 (LLMWEAVTV) peptides respectively.

In our study, we selected HLA-A2 restricted immunodominant peptides derived from the capsid protein VP1 of JCV and BKV (JCV-VP1-p100: ILMWEAVTL and BKV-

VP1-p108: LLMWEAVTV). These 9mer peptides were homologous for both viruses except at the first and the last position amino acid residues (Figure 17). After MLPC, each peptide-stimulated $CD8^+$ T cell responses were measured by both JCV and BKV peptide-specific MHC-tetramers. Peptide-stimulated T cells were measured equally when both JCV and BKV-specific MHC-tetramers were used in a criss-cross manner. This indicated that there was high TCR cross-reactivity between T cells specific for both JCV and BKV (Figure 18). We also observed functional cross-reactivity among these T cells.

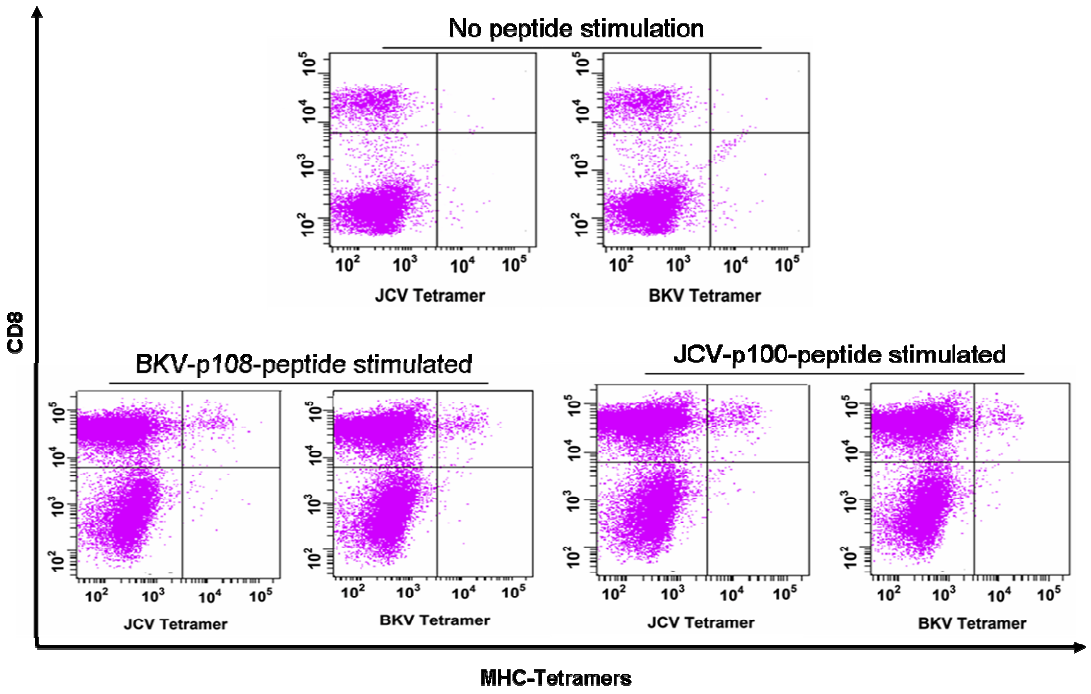


Figure 18: TCR cross-reactivity between T cells specific for JCV and BKV peptides: $CD8^+$ T cells were stimulated with JCV and BKV immunodominant peptides respectively. After a week of culture, cells were double-stained for $CD8^+$ T cells and MHC-tetramers. Complete medium (no peptide) stimulated $CD8^+$ T cells were used as negative control.

3.5 HLA-typing of HDs

Allele-specific antibodies were initially used to type HDs for HLA-A2 and HLA-B7. In this context, K562 cells transfected with HLA-A2 and HLA-B7 were used as positive controls in flow cytometry. Additionally, K562 cells transfected with HLA-A1, HLA-B7 and HLA-B8 were also used as internal controls to exclude cross-reactivity. We observed that HLA-A2 allele-specific antibody (BB7.2) staining was distinct and clear and did not show any cross-reactivity to other HLA-alleles (HLA-A1, HLA-B7, HLA-B8) (Figure 19 A). However, using the HLA-B7 allele-specific antibody, we observed that staining was not of high intensity and it also showed cross-reactivity to the K562-A1 transfectants (Figure 19 B). To solve this problem SSP-PCR protocol was standardized and sequence specific primers were selected for HLA-A2 (Bunce 2003) and HLA-B7 (Hollsberg 2002). Primers were analyzed using primer-BLAST and product length was determined for HLA-A2 (494bp) and HLA-B7 primers set (119bp). These primers were then tested for the optimum annealing temperature using the gradient PCR method. According to Tm of primer sets, the temperature for gradient PCR was set. At 64.8^0C using DNA obtained from K562-B7 cells as a positive control and no DNA as a negative control, we observed a clear band of 119bp by gel electrophoresis (Figure 20 A). Using this temperature as annealing temperature, a PCR protocol was established and HDs were typed for the HLA-B7 allele. We detected a clear band of 119bp with DNA from K562-B7 cells with no cross-reactivity across HLA class I when SSP-PCR was used (Figure 20 B). Likewise, protocol for HLA-A2 was also established using SSP-PCR.

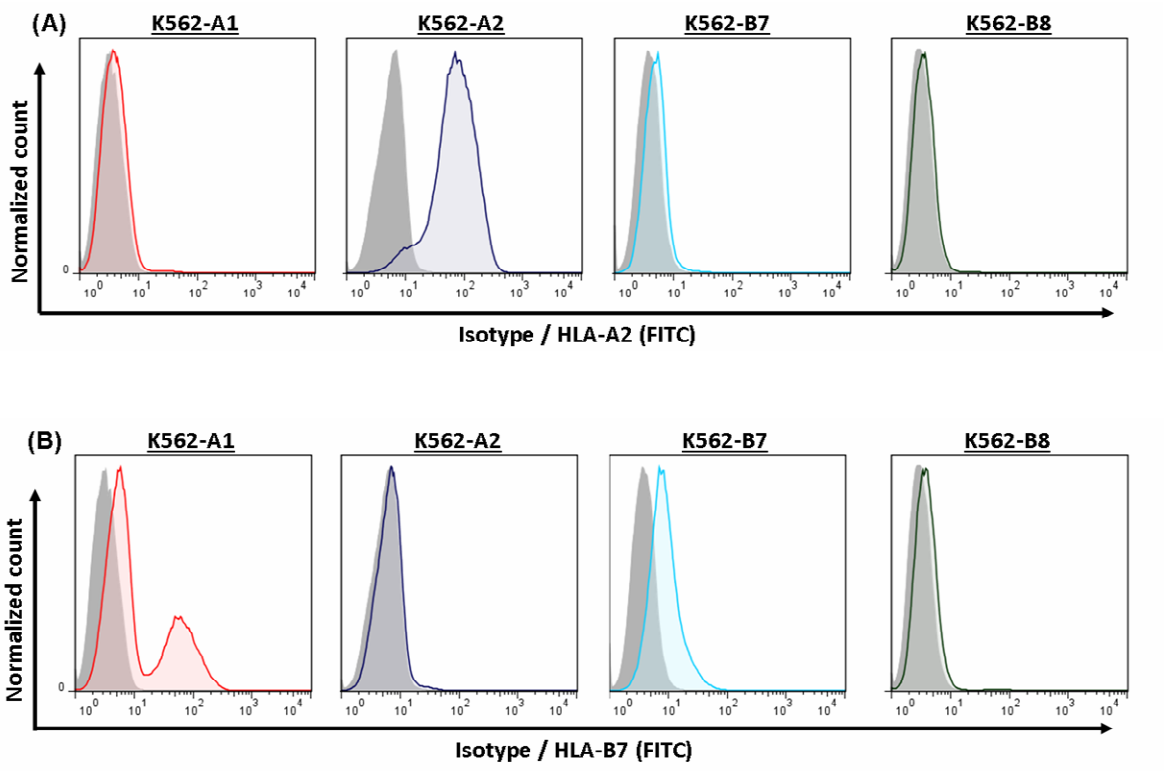


Figure 19: HLA-typing by flow cytometry: Histograms showing overlays of isotype control and allele-specific fluorescent antibody staining. K562 transfectants for HLA-A1, HLA-A2, HLA-B7 and HLA-B8 were stained with (A) isotype control and HLA-A2-specific antibody (BB7.2), (B) isotype control and HLA-B7-specific antibody respectively.

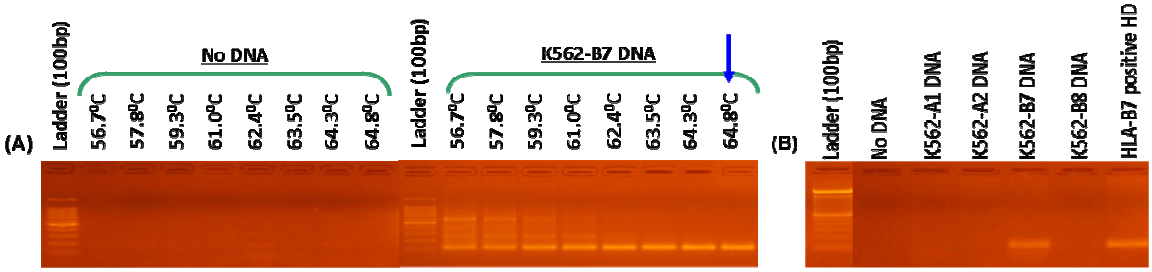


Figure 20: Gel electrophoresis of SSP-PCR products: (A) Gradient PCR using K562-B7 DNA and no DNA as positive and negative control respectively. The gradient temperature was set according to the calculation of Tm of the primer set. (B) PCR at 64.8^{0}C using all the transfected K562 cell lines and an HLA-B7 positive healthy donor.

In summary, both methods of antibody mediated flow cytometry and PCR were employed for HLA-typing of HDs. However, due to unwanted cross-reactivity in flow cytometric approach we used latter approach for specific HLA-typing, especially for HLA-B7 allele. Using both approaches, it was observed that out of total donors

tested 50% were HLA-A2 positive, 30% were HLA-B7 positive and 13% were positive for both HLA-A2 and HLA-B7 allele.

3.6 Pre-screening of JCV-VP1-specific T cells in HDs

At present, the measurement of anti-VLP antibodies and JCV-DNA in the urine, constitute the two methods used to determine a present or past JCV infection or reactivation. Combining these two methods almost 70-90% of the human population was shown to be seropositive. However, it is not clear whether a JCV seropositive individual also constitute a JCV-specific positive T cell response which is marked by circulating T cells (Jelcic, Aly et al. 2013). Here we tried to screen HDs for positive T cell response prior to detailed analysis.

HDs pre-screening was performed after one week of MLPC. Pre-screening of non-HLA-A2 positive donors was performed by using a JCV-VP1-peptide pool in CFSE proliferation and IFN-γ ELISPOT assays. To screen HLA-A2 positive HDs, two immunodominant peptides derived from JCV-VP1 (p36 and p100) and corresponding MHC-tetramers were also used. We observed that using different experimental technique every two out of ten HDs were screened positive for JCV-VP1-specific T cells.

When comparing different read-outs for pre-screening, we observed that even if CFSE proliferation assay was sensitive and convenient to use, results were not always equivalent to IFN-γ ELISPOT assay (Figure 21 A, B). To compare in MLPC, p100-specific T cell responses were measured in two HLA-A2 positive HDs using proliferation and ELISPOT assay. The numbers of IFN-γ secreting T cells were comparable in both donors. However, a difference was found between populations

which undergone proliferation (Figure 21 A, B). Similar results were also obtained using p36-peptide (data not shown).

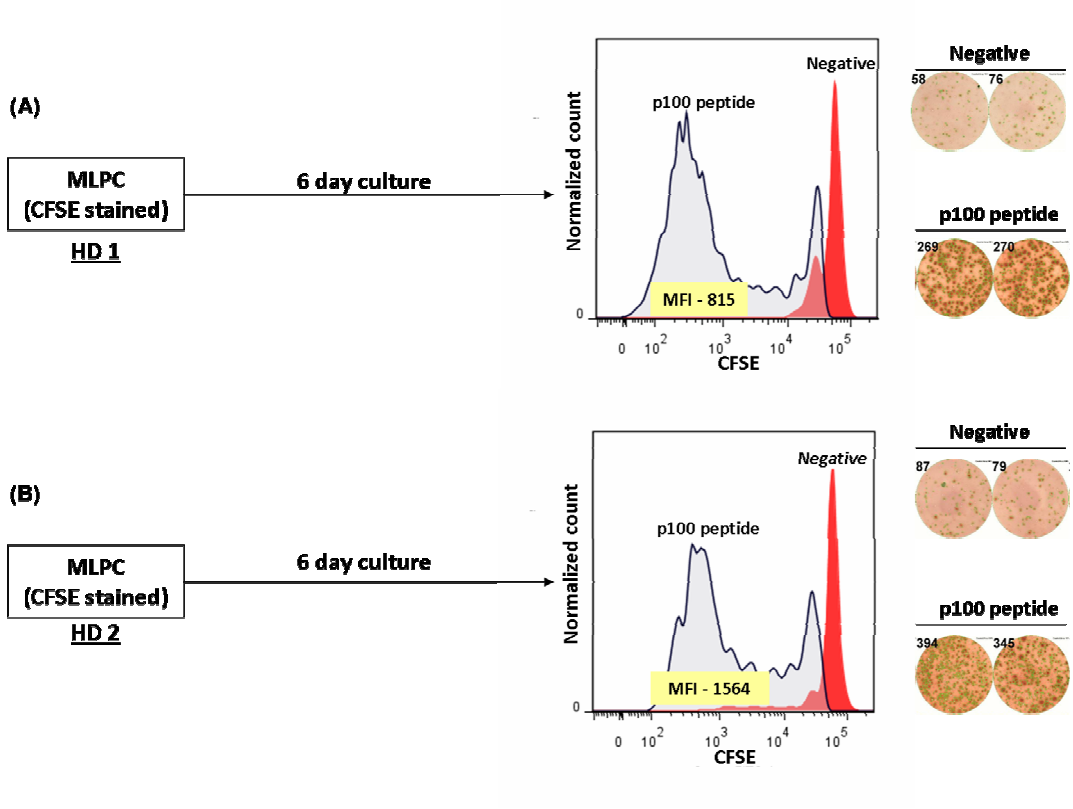


Figure 21: Pre-screening of HDs, based on proliferation and IFN-γ secretion: MLPC was performed using p100-peptide. Here, T cells were also labelled with CFSE. After 6 days of culture, one half of T cells were stained with anti-CD8 antibodies and measured for CFSE labelling in flow cytometry. Another half was restimulated for IFN-γ secretion in the ELISPOT assay. (A) HD1 has undergone more rounds of proliferation (MFI: 815). (B) HD2 has undergone less rounds of proliferation compared to HD 1 (MFI: 1564). MFI: mean fluorescence intensity.

Overall, we could analyze HDs for JCV-specific T cells irrespective of HLA restriction based on the peptide-specific TCR, proliferation and IFN-γ secretion properties. Also, we found that for pre-screening purpose, information about proliferation by CFSE alone was insufficient so this method was always combined with either IFN-γ secretion or MHC-tetramers.

3.7 Processing of JCV-VP1-VLP

In order to find out whether the responding antigenic peptides were naturally processed, we used JCV-VLP as antigens for stimulation. JCV-VLP were a generous gift from Prof. Alma Gedvilaite (Vilnius University). VLP is a multimeric protein made of refolded recombinant VP1 and has a morphologic and structural similarity to the natural JCV capsid (Chang, Fung et al. 1997; Zielonka, Gedvilaite et al. 2006).

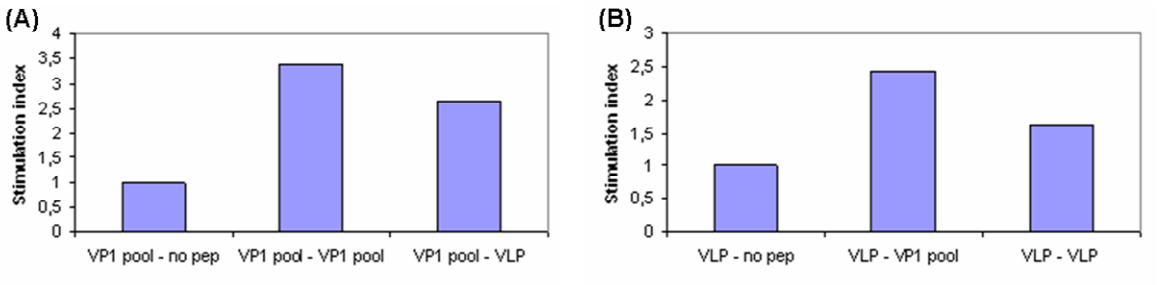


Figure 22: Analysis of JCV-VLP processing: (A) MLPC was performed using the VP1-peptide pool as stimulus. (B) MLPC was performed using JCV-VLP as stimulus. First, second and third bar in both graphs show restimulation performed with complete medium (no peptide), VP1-peptide pool and JCV-VLP.

We wondered whether JCV-VLP-stimulated T cells could recognize JCV-VP1-peptide pool and vice-versa. To this end to assess this problem, $CD8^+$ T cells were stimulated with JCV-VP1-peptide pool (Figure 22 A) and VLP (Figure 22 B) in parallel. Restimulation was performed using both the VP1-peptide pool and VP1-VLP. Complete medium served as negative control. We observed that VP1-peptide pool stimulated T cells showed high T cell responses after restimulation with JCV-VLP (Figure 22 A). Similar T cell responses were observed with VLP-stimulated cells when restimulation was effective by the VP1-peptide pool (Figure 22 B). In both settings, a positive signal in the ELISPOT assay demonstrated that VP1-protein antigen was processed and the peptides resulting from the proteasomal cleavage

process were successfully presented via HLA class I molecules to $CD8^+$ T cells. Moreover, equally high T cell responses were observed after stimulation and restimulation with the same antigen (Figure 22 A, B).

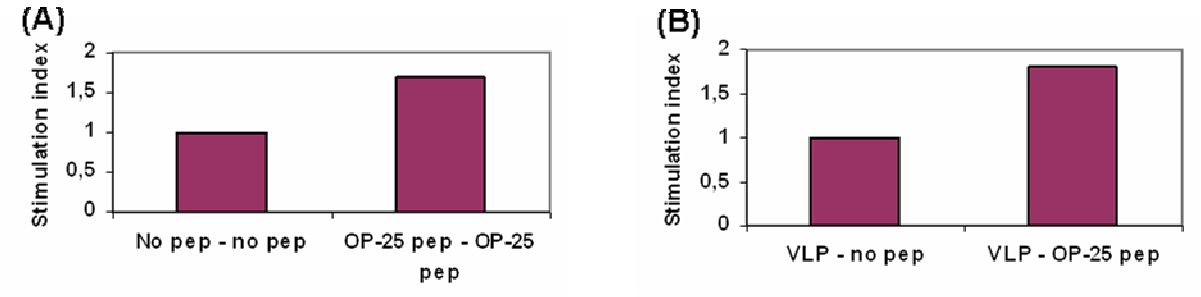


Figure 23: Analysis of VLP processing using the OP25 peptide: (A) MLPC was performed using complete medium (no peptide) versus the OP25 peptide as a stimulus. (B) In MLPC JCV-VLP served as stimulus. The first and the second bar in both graphs show restimulation performed with complete medium and the OP25 peptide.

Furthermore, using an individual 15mer peptide designated OP25 which encompassed JCV-TCE p100, we confirmed the natural processing of JCV-VLP. In the experiment shown in Figure 23, two MLPCs were performed in parallel, the first MLPC was stimulated by the OP25 peptide and the second MLPC by JCV-VLP. Both cultures were thereafter restimulated with OP25 peptide in the ELISPOT assays. We observed positive T cell responses to OP25 when the peptide was used for both stimulation and restimulation (Figure 23 A). Equally good T cell responses were observed when VLP-stimulated T cells were restimulated with OP25 peptide (Figure 23 B). Responses to the individual peptide by JCV-VLP-stimulated T cells confirmed that the VLP is naturally processed and VLP-derived peptides are presented on HLA class I molecules.

Overall, we observed that JCV-VLP are processed and presented efficiently by APCs in MLPC. Furthermore, processing was confirmed by using immunodominant JCV-TCEs.

3.8 Assessment of novel JCV-VP1 CD8$^+$ T cell epitopes (TCEs)

3.8.1 Novel T cell epitopes (TCEs) in HDs

In order to map T cell antigens, a comprehensive approach using 19 JCV-VP1-peptide sub-pools were employed in a matrix setup (discussed in 3.2.2.3, Figure 13). All HDs were first HLA-typed by flow cytometry and SSP-PCR methods (discussed in 3.5). Donors, who were positive for HLA-A2 and HLA-B7 were selected and analyzed for T cell reactivity to the JCV-VP1-peptide pool. Samples from HDs showing positive immune responses were then used for further identification of potential TCEs. A systematic scheme for identification of TCEs to JCV-VP1 is displayed in Figure 24. The first step was to identify dominant T cell specificities using the peptide matrix pool approach (Figure 24 A). Here, the stimulation (at day 0) was initiated by JCV-VLP. This was a very important step, as it confirmed that the stimulating antigenic peptides were not artificial but naturally processed. After identification of a particular T cell specificity, restriction analysis was performed using HLA-transfectants loaded with individual 15mer peptides (Figure 24 B). For this purpose, T2 cells and K562 cells transfected with HLA-A2 or HLA-B7 alleles were used as APCs. To further characterize the minimal epitope, 9mer peptides were predicted by the use of the SYFPEITHI software (http://www.syfpeithi.de/), synthesized and then tested individually (Figure 24 C).

By IFN-γ ELISPOT assays, strength of T cell responses was estimated using stimulation index (S.I) which was calculated for each positive signal after dividing the number of dots in the experimental wells in the 96-well plate with the negative control wells. Based on this index, T cell responses were classified as weak (between 2 and 3), moderate (between 3 and 4) or strong (≥4).

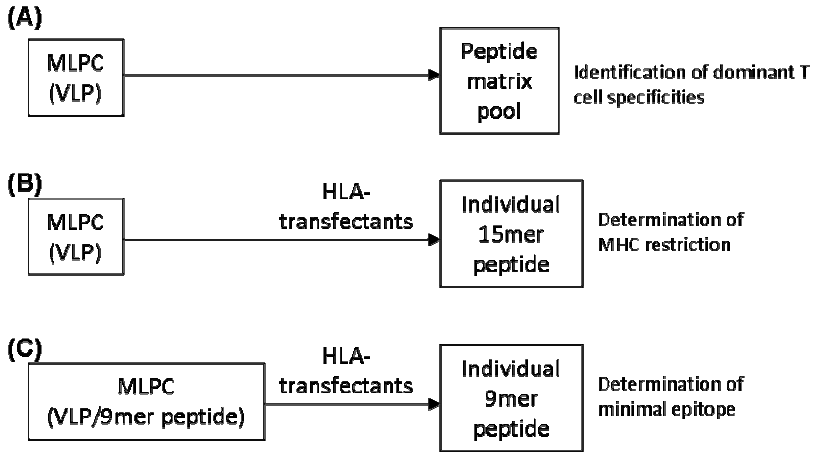
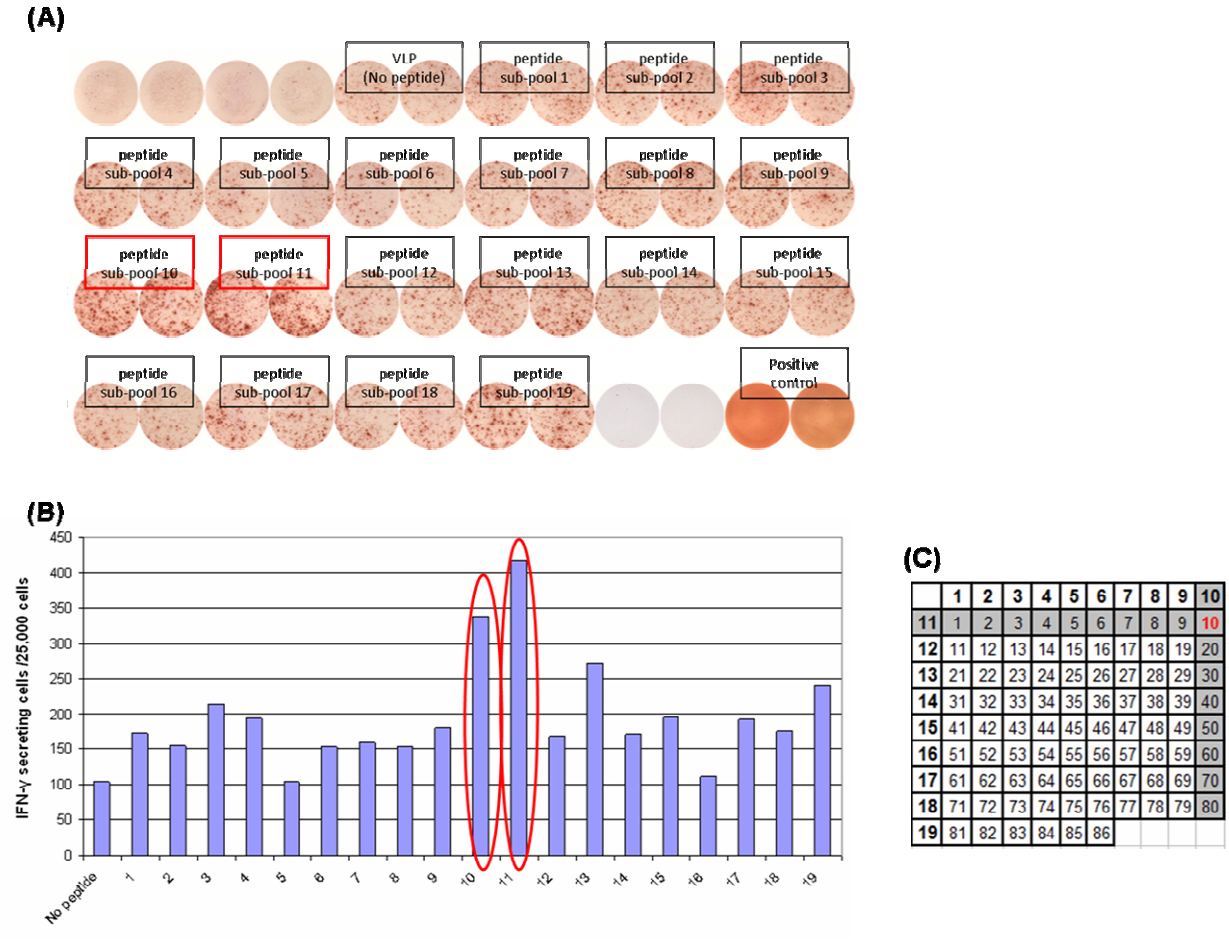


Figure 24: Systematic scheme of T cell epitope mapping: (A) Identification of T cell specificities using the peptide matrix pool approach. (B) Determination of the restriction molecule using selected individual 15mers in combination with HLA-transfectants. (C) Determination of the fine epitope specificities using predicted 9mer peptides.

The first step, i.e. the identification of T cell specificities using the peptide matrix pool is a very crucial one. A representative experiment employing peptide matrix pool stimulation is depicted in Figure 25. In this representative example, JCV-VLP-stimulated $CD8^+$ T cells were restimulated with 19 peptide matrix sub-pools in an ELISPOT plate with each restimulation in duplicate. Complete medium and SEB stimulation served as negative and positive controls, respectively. Two positive responding peptide sub-pools (sub-pools 10 and 11) were identified on the basis of number of IFN-γ secreting cells (red ellipses) (Figure 25 A, B). In a further step, the common peptide present in both peptide sub-pools was selected with the help of

matrix grid (Figure 25 C). This 15mer peptide (in this experiment: OP10) was used in subsequent experiments to analyze the T cell reactivity.



	1	2	3	4	5	6	7	8	9	10
11	1	2	3	4	5	6	7	8	9	10
12	11	12	13	14	15	16	17	18	19	20
13	21	22	23	24	25	26	27	28	29	30
14	31	32	33	34	35	36	37	38	39	40
15	41	42	43	44	45	46	47	48	49	50
16	51	52	53	54	55	56	57	58	59	60
17	61	62	63	64	65	66	67	68	69	70
18	71	72	73	74	75	76	77	78	79	80
19	81	82	83	84	85	86				

Figure 25: T cell epitope screening using matrix peptide sub-pools: (A) ELISPOT wells showing negative and positive controls as well as wells stimulated by nineteen different peptide sub-pools in duplicate. Experimental wells stimulated with peptide sub-pools 10 and 11 were marked as positive results. (B) Histogram with bars representing IFN-γ secreting cells per 25,000 cells. Based on the T cell responses, two matrix peptide sub-pools were selected (red ellipses) and (C) the corresponding individual 15mer peptide common to both sub-pools was identified (OP10) in the matrix grid.

Retrieval of previously known JCV-VP1 T cell epitopes

Using the matrix approach, we were able to confirm two previously known HLA-A2 restricted JCV-VP1 epitopes p36 and p100. Using the two 15mer peptides OP9 and OP25 containing these epitopes, we found T cell reactivity in more than 4 out of 12

donors (Table 3). Additionally, p36 and p100-peptides were used in all 22 HLA-A2 positive donors tested and we found that 31% donors were positive for p36 and 41% donors were positive for p100-peptides (Table 5).

Identification of novel T cell specificities

In addition to reactivity to p36 and p100-peptides, we found several other specificities in HLA-A2 positive donors (Table 3). Some of the T cell responses to a particular antigenic peptide were shared by more than three HDs (≥25% HDs), for e.g. the peptides OP9, OP25, OP28, OP29, OP72 and OP78. In four different donors, stimulation by the two 15mers OP72 and OP78 resulted in positive signals. Especially donor#4318 showed a strong T cell response to the OP72 peptide in three independent experiments. Experiments towards the characterization of 9mers predicted from OP72 and OP78 are ongoing. The peptide OP29 was another 15mer peptide with strong signals in 33% of the donors we tested. Using the SYFPEITHI software, the 9mer peptide named p113 was predicted. The peptide was synthesized and used in experiments with twelve HLA-A2 positive donors. We observed that 3 out 12 donors tested were positive for T cell responses to peptide p113 including the HD#0132 who showed an exceptionally strong T cell response.

Table 3: Antigenic peptides tested in **HLA-A2** positive healthy donors (HDs). TCE of 15mers OP9 and OP25 peptides was described (see Table 1). All other antigenic peptides described here are novel. The restriction molecules were predicted using SYFPEITHI software (http://www.syfpeithi.de/). ID represents the last three to four digits from the blood bank code for the HDs.

JCV-VP1-peptides	ID	*In silico* predicted T cell epitope	Experimental T cell reactivity
GVDSITEVECFLTPE (OP9)	0196	S **I** T E V E C F **L**	Weak
	6551		Moderate
	2004		Moderate
	4195		Moderate
	4318		Moderate
MLPCYSVARIPLPNL (OP20)	0196	V **A** R I P L P N **L**	Moderate
CGNILMWEAVTLKTE (OP25)	0196	I **L** M W E A V T **L**	Moderate
	4895		Moderate
	0132		Moderate
	2004		Moderate
KTEVIGVTSLMNVHS (OP28)	4895	T **E** V I G V T S **L**	Moderate
	2004		Weak
	5728		Moderate
IGVTSLMNVHSNGQA (OP29)	0196	I **G** V T S L M N **V**	Strong
	4895		Moderate
	2004		Weak
	5728		Moderate
PVECWVPDPTRNENT (OP50)	0196	W **V** P D P T R N **E**	Moderate
PTRNENTRYFGTLTG (OP52)	0196	E **N** T R Y F G T **L**	Moderate
VDVCGMFTNRSGSQQ (OP65)	0196	G **M** F T N R S G **S**	Weak
KRRVKNPYPISFLLT (OP72)	4318	K **N** P Y P I S F **L**	Strong
	2004		Moderate
	0065		Weak
	5728		Moderate
KNPYPISFLLTDLIN (OP73)	4318	P **I** S F L L T D **L**	Moderate
VDGQPMYGMDAQVEE (OP78)	0132	P **M** Y G M D A Q **V**	Moderate
	5728		Moderate
	0014		Weak
	0196		Weak
PMYGMDAQVEEVRVF (OP79)	0132	G **M** D A Q V E E **V**	Moderate
GTEELPGDPDMMRYV (OP83)	0196	E **L** P G D P D M **M**	Moderate
IGVTSLMNV (p113)	4895	I **G** V T S L M N **V**	Moderate
	132		Strong
	0196		Weak
ILMEWEAVTL (p100)	0992	I **L** M W E A V T **L**	Strong
	4090		Strong
	0132		Moderate
	0207		Strong
	2564		Strong
	2004		Moderate
	4895		Strong
	4195		Moderate
	4318		Moderate
SITEVECFL (p36)	0196	S **I** T E V E C F **L**	Strong
	4129		Weak
	2564		Moderate
	2004		Weak
	4195		Strong
	4318		Moderate
	4895		Weak

In HLA-B7 positive donors, we observed moderately positive T cell responses to 15mer peptides at a frequency of one out of ten HDs (Table 4). In an attempt to characterize TCEs in one donor#4196, who was positive to the 15mer peptide OP76 (Table 4), the predicted 9mer was synthesized and tested. However, corresponding 9mer peptide (p306) stimulus gave no T cell responses (data not shown). In another experiment, donor#4278 showed exceptionally strong secretion of IFN-γ when T cells were stimulated with 9mer peptide p244 (Table 4). These results were reproducible in independent experiments and none of the other nine donors showed T cell responses to this peptide.

Table 4: Antigenic peptides tested in ten **HLA-B7** positive healthy donors (HDs). All peptides described here are novel. The restriction molecules were predicted using SYFPEITHI software (http://www.syfpeithi.de/). ID represents the last three to four digits from the blood bank code for the HDs.

JCV-VP1-peptides	ID	*In silico* predicted T cell epitope	Experimental T cell reactivity
TPEMGDPDEHLRGFS (OP12)	1608	D **P** D E H L R G **F**	Weak
GDPDEHLRGFSKSIS (OP13)	4106	D **P** D E H L R G **F**	Strong
ESDSPNRDMLPCYSV (OP18)	4169	S **D** S P N R D M **L**	Moderate
PNLNEDLTCGNILMW (OP23)	4106	E **D** L T C G N I **L**	Moderate
	4196		Weak
HDNGAGKPVQGTSFH (OP33)	4106	K P **V** Q **G** T S F **H**	Weak
VLFNYRTKYPDGTIF (OP40)	4106	R **T** K Y P D G T **I**	Moderate
ENTRYFGTLTGGENV (OP53)	4106	E **N** T R Y F G T **L**	Weak
	4196		Moderate
PVLHITNTATTVLLD (OP57)	4106	I **T** N T A T T V **L**	Moderate
PLCKGDNLYLSAVDV (OP62)	4169	L **C** K G D N L Y **L**	Moderate
KRRVKNPYPISFLLT (OP72)	4169	N **P** Y P I S F L **L**	Moderate
LINRRTPRVDGQPMY (OP76)	4196	T **P** R V D G Q P **M**	Moderate
RVFEGTEELPGDPDM (OP82)	1608	R **V** F E G T E E **L**	Moderate
GPLCKGDNL (p244)	4278	G **P** L C K G D N **L**	Strong
NPYPISFLL (p290)	1608	N **P** Y P I S F L **L**	Moderate
	4106		Weak
VARIPLPNL (p83)	1608	V **A** R I P L P N **L**	Moderate
	4106		Weak
	4195		Moderate

In summary, we observed that among all HLA-B7 restricted T cell specificities given in Table 4, two out of ten donors showed T cell responses to the peptides OP23,

OP53 and p290, whereas three out of ten donors tested were positive for the 9mer peptide p83. The p83-peptide was also shown to be immunogenic in one patient sample (discussed in 3.8.2).

Based on the frequency and strength of T cell responses in HDs, some of the candidate TCEs including p36 and p100 were summarized in Table 5. It was observed that the frequency of positive donors for all candidate TCEs were in the range of 25% to 41% with the top score for p100-peptide. To estimate the relative strength of T cell stimulation by each antigen peptide in a responding donor, we calculated the magnitude (index) of T cell responses. For example, a strong, moderate and weak T cell response to a particular antigenic peptide in a single donor was given a magnitude (index) of 1, 0.66 and 0.33 respectively. Based on this calculation, it was observed that in a positively responding HD, T cell responses to p100-peptide were very high while T cell responses to OP78 peptide were of lowest magnitude.

Table 5: Donor frequencies specific to potential TCEs.

JCV-VP1-peptides	Total donors tested	Positive donors	Magnitude (index) of T cell response (maximum 1)
p83	10	3 (30%)	0.55
OP29	12	4 (33%)	0.66
OP72	12	4 (33%)	0.58
OP78	12	4 (33%)	0.50
p113	12	3 (25%)	0.66
p100	22	9 (41%)	0.86
p36	22	7 (31%)	0.59

In summary, we could observe several novel candidate HLA class I-TCEs in HLA-A2 and HLA-B7 positive HDs using our approach. Additionally, previously described HLA-A2 restricted immunodominant peptides were confirmed. Using JCV-VLP as

primary stimulus, we demonstrated that all newly defined TCEs were naturally processed.

3.8.2 Novel T cell epitopes (TCEs) in a patient

In addition to HDs, we had the opportunity to analyze a patient for polyoma-reactive $CD8^+$ T cells. The HLA class I and class II typing is shown in Table 6.

Table 6: HLA-typing of patient blood sample.

HLA-A		HLA-B		HLA-C		DRB1*		DQB1*		DQA1*	
3	29	7	44	W*07	W*16	0101	0701	05	02	0101	0201

This patient had a history of a severe HC. Due to his previous exposure to BKV, we decided to analyze the T cell response to BKV-VLP. In parallel, cross-reactivity to JCV was also measured using stimulation with JCV-VLP, the JCV-VP1-peptide pool and a pool of HLA-B7-binding peptides. HLA-B7-binding peptides were considered for two reasons. Firstly, a more comprehensive approach (e.g. individual peptides of VP1-protein) was not possible due to limited availability of patient sample. Secondly, our lab has a long-standing interest in the HLA-B7 molecule and T cell specificities restricted to HLA-B7 (clinical study: CMVpp65 vaccination in patients after stem cell transplantation).

To this end, the amino acid sequence of the JCV-VP1-protein was screened and six nonamer HLA-B7-binding peptides were predicted on the basis of prediction scores using two programs SYFPEITHI (http://www.syfpeithi.de/) (score of more than 18) and BIMAS (http://www-bimas.cit.nih.gov/molbio/hla_bind/) (score of more than 180). In MLPCs, $CD8^+$ T cells were stimulated using DCs loaded with JCV-VLP, the JCV-VP1-peptide pool and a pool of HLA-B7-binding peptides. In IFN-γ secretion assays

we observed high T cell responses to both BKV-and JCV-VLP (Figure 26 A, B). Moreover, we observed equally high T cell responses to both the VP1-peptide pool and the pool of HLA-B7-binding peptides derived from JCV (Figure 26 A, B). A positive response to the pool of HLA-B7-binding peptides suggests the presence of possible T cell determinants in this pool.

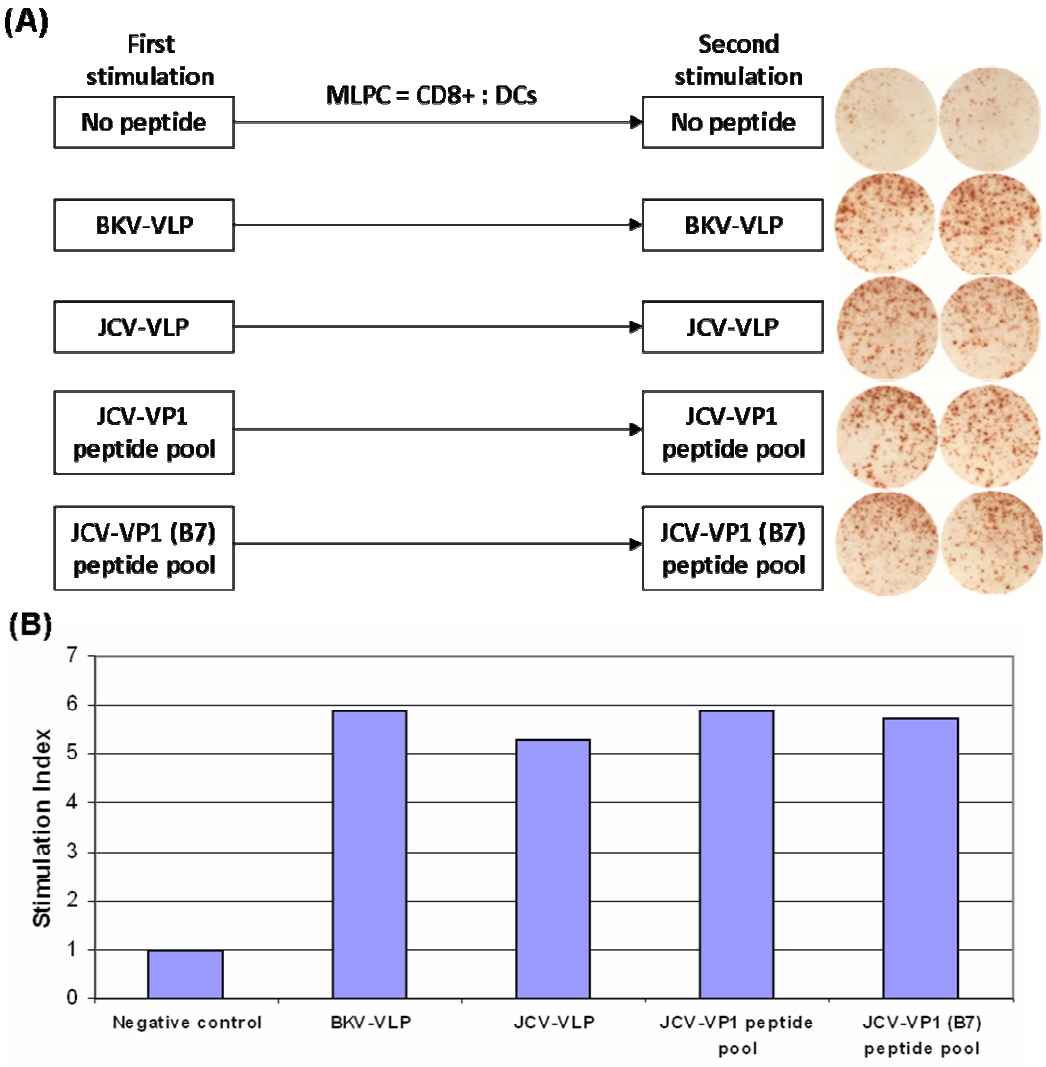


Figure 26: T cell responses to JCV and BKV in a HLA-B7 positive patient: IFN-γ ELISPOT assays were performed using autologous DCs loaded with complete medium, BKV-VLP, JCV-VLP, the JCV-VP1-peptide pool and a B7-peptide pool. IFN-γ secretion by T cells as original data in ELISPOT plate wells as well as histogram with bars indicating the respective S.I. (S.I.: for further details see page 67)

To identify further which one of the HLA-B7-binding peptides induced a positive $CD8^+$ T cell response, MLPCs were performed using individual peptides. First, $CD8^+$ T cells were stimulated using DCs loaded with JCV-VLP. Then, $CD8^+$ T cells were restimulated in ELISPOT assays using JCV-VLP, the pool of HLA-B7-binding peptides and all the HLA-B7-binding peptides individually. In the ELISPOT assays, complete medium and SEB served as negative and positive controls respectively. We observed that there were significantly high T cell responses to JCV-VLP. Additionally, strong T cell responses were measured to the individual peptide p83 while moderate T cell responses were observed to p306-peptide (Figure 27 A, B). Other peptides showed rather low or no IFN-γ secretion. Since the first stimulation was effective through JCV-VLP loaded DCs, it was evident that all responding peptides were naturally processed.

We also observed T cell responses to the pool of HLA-B7-binding peptides. However, the T cell response was low when compared to the individual peptides and VLP (Figure 27 A, B). The lower response might be due to peptide competition (six peptides in a pool) for binding to the presenting HLA molecules.

In conclusion, in a patient with a previous history of HC we observed high T cell responses to both BKV-and JCV-VLP which indicates the possibility of cross-reactivity among BKV-and JCV-specific T cells. Furthermore, we found T cell specificities to the two predicted HLA-B7-binding peptides p83 and p306. Both peptides were demonstrated to be naturally processed.

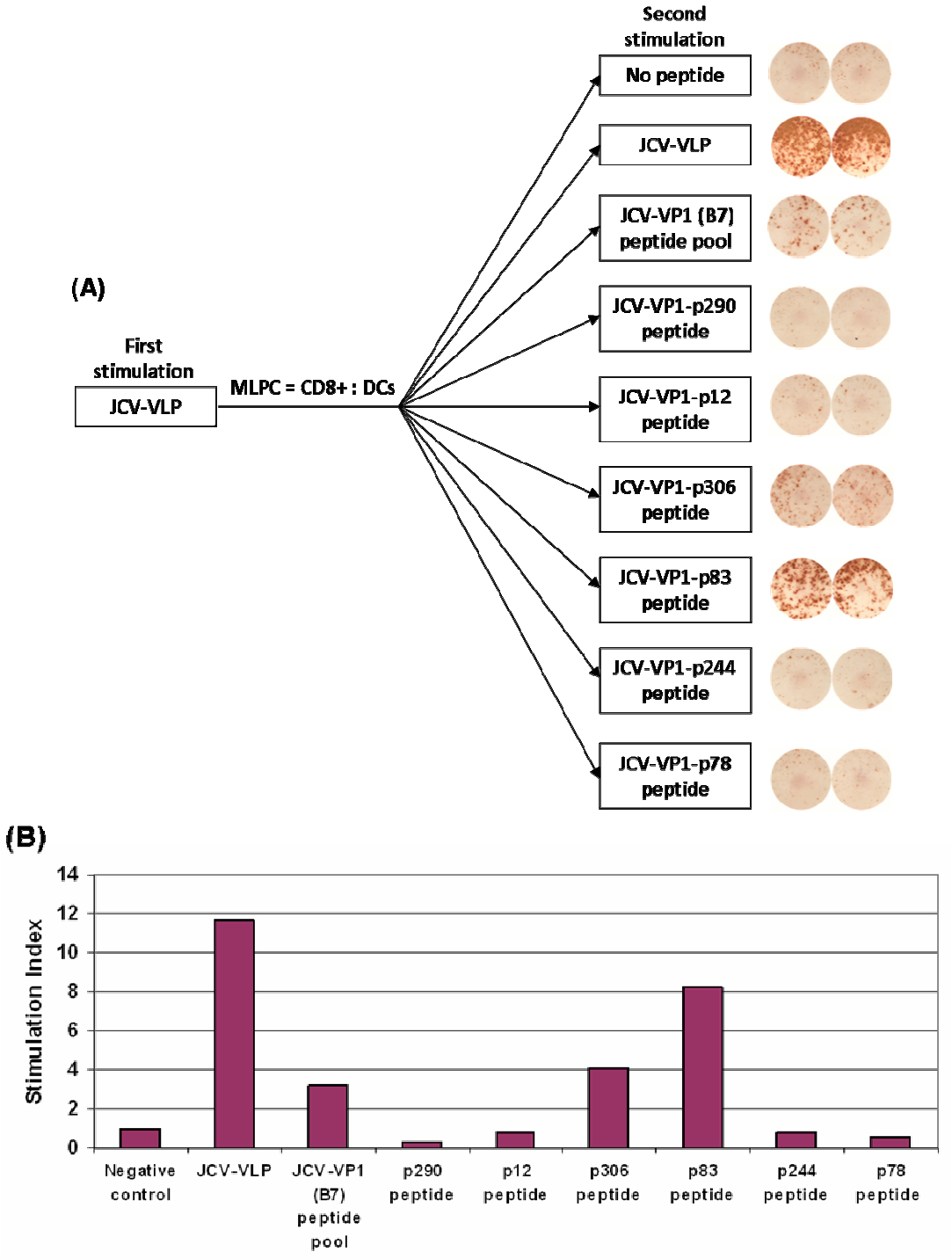


Figure 27: TCE mapping in a patient: IFN-γ ELISPOT assays were performed using autologous DCs loaded with complete medium, JCV-VLP, the pool of HLA-B7-binding peptides and six individual HLA-B7-binding peptides. IFN-γ secretion by T cells as original data in ELISPOT plate wells as well as histogram with bars indicating the respective S.I. (S.I.: for further details see page 67).

4 DISCUSSION

Frequencies of JCV-specific T cells

Ex vivo stimulation using an array of peptides constitutes a widely used method to detect precursor frequencies (Sun, Burton et al. 2000; Pittet, Zippelius et al. 2001; Anthony and Lehmann 2003; Speiser, Pittet et al. 2004; Guerreiro, Na et al. 2010; Li Pira, Ivaldi et al. 2010). To assess the precursor frequency specific to JCV, we performed *ex vivo* IFN-γ secretion assays using VP1-peptide pool in eight HDs. Using 100,000 PBMCs as effector cells, stimulation was performed for almost 24 hrs but no T cell reactivity to JCV-VP1-peptide pool was observed. To assess the precursor T cell number one week expansion step was performed and relative T cell frequencies were measured (Table 2). Here, we found that after *in vitro* stimulation, frequencies towards JCV were lower when compared to other viruses like CMV and the influenza virus (van Aalderen, Remmerswaal et al. 2013) (Table 2). One possible explanation for this low frequency of JCV-specific T cells is the replication pattern of persistent viruses in an immunocompetent individual. The primary infection of JCV occurs during childhood and once the viruses are cleared, it remains latent in sites like the urogenital tract or CNS. In these anatomical compartments, viruses replicate occasionally (Polo, Perez et al. 2004). Also, JCV normally is not found in blood (viremia) (Koralnik, Boden et al. 1999). Taken together these factors might be the reason for the generation of a rather low number of memory T cells. Nevertheless, this low number of memory T cells is potent enough to control JCV-reactivation in the immunocompetent host.

Optimization of the T cell expansion protocol

Due to very low precursor frequency, *ex vivo* monitoring of JCV-specific T cells is very difficult. Therefore, an *in vitro* expansion protocol was necessary. Optimization of such a protocol was crucial, especially for the successful stimulation of low-affinity T cells. Here, we compared different protocols varying in the type of APCs like monocytes, PBMCs, and depleted fraction of $CD8^+$ T ($CD8^-$) cells. Autologous PBMCs as APCs has been already reported earlier (Fu, Freed et al. 2001; Binggeli, Egli et al. 2007; Ginevri, Azzi et al. 2007; Marzocchetti, Lima et al. 2009). Using immunodominant peptides-derived from two different viruses, we observed an almost 5-to 8.5-fold increase in the expansion of peptide-specific $CD8^+$ T cells when $CD8^-$ was used as APCs at a four times higher ratio (Figure 8 and Chapter 3.2.1). A $CD8^+$ to $CD8^-$ cell ratio of 1:4 was previously optimized in our lab. Here, $CD8^-$ cells not only served as autologous feeder cells but also as APCs. It has been observed that in the absence of feeder cells, $CD8^+$ T cells were unable to expand (Al-Shanti and Aldahoudi 2007). Moreover, $CD8^-$ cells were irradiated which ensured that this fraction would not proliferate and only $CD8^+$ T cells would contribute in the overall expansion. The importance of irradiated autologous feeder cells has been discussed in one study where it is proposed that irradiating <50% of PBMCs contributes in high proliferation of non-irradiated PBMCs (Jackson, Dimopoulos et al. 2004). Additionally, to support the T cell culture, two recombinant cytokines IL-2 and IL-7 were used in our protocol. The role of IL-2 in T cell proliferation has been already demonstrated (Friedmann, Migone et al. 1996; Ahmed, Grimes et al. 1997). The selection of IL-7 was based on the fact that all memory T cells express IL-7R-α on their surface (He, Mahmood et al. 2003; de Bree, Heidema et al. 2005).

ELISPOT assays

To identify *in vitro* stimulated T cells even at low frequency we used ELISPOT assays as a sensitive read-out system (McCutcheon, Wehner et al. 1997). For the optimization we used different types of APCs at different ratios (Chapter 3.2.2.1). Cell lines like K562 and T2 were confirmed to be as good as autologous PBMCs to restimulate $CD8^+$ T cells in ELISPOT plates (Chapter 3.2.2.1). SEB, a super-antigen which has been shown to stimulate T cells non-specifically, served as a positive control (Boshell, McLeod et al. 1996; McLeod, Walker et al. 1998). SEB was preferred over PHA because in our hands it gave more reproducible results than PHA: SEB was used to stimulate T cells in the presence of APCs as it has been reported that in the absence of co-stimulation, SEB causes T cells to undergo apoptosis instead of proliferation (Boshell, McLeod et al. 1996; McLeod, Walker et al. 1998).

Peptide length

The effect of peptide length on the initiation of T cell responses is still under debate (Zwaveling, Ferreira Mota et al. 2002; Sercarz and Maverakis 2003; Bijker, van den Eeden et al. 2008). In order to analyze whether HLA-transfectants could efficiently process longer peptides (15mer) and stimulate T cell responses, we used 9mer and 15mer peptides derived from two different viral antigens. It was observed that 15mer peptides can be efficiently processed and presented by both PBMCs and cell lines (Chapter 3.2.2.2 and 3.2.2.3). This information was very crucial for establishing strategy for restriction analysis of antigenic peptides using HLA-transfectants (Figure 24).

Using titrations of 9mer and 15mer peptides, we observed that 9mer peptides are superior in initiating T cell responses. At low concentrations, the dose-response curve of 15mer peptides was still in a linear phase while it was already in plateau with the same concentration of 9mer peptide (Figure 12, 16) (Kiecker, Streitz et al. 2004). This indicates that for the initiation of T cell responses, higher concentrations of 15mer peptides is required when compared to 9mer peptides (Figure 12).

Characterization of JCV-VP1-specific T cells

In order to characterize T cells specific for JCV-peptides, we selected HDs screened positive for nonamer JCV-VP1-p100 and CMVpp65-peptide. Using four different concentrations of JCV-VP1 and CMVpp65-peptides (10 ng/ml, 100 ng/ml, 1 µg/ml and 10 µg/ml), the relative affinity or avidity of peptide-specific T cells were determined. MHC-tetramers were used to measure the peptide-specific T cells. It has been reported that the affinity of TCRs can be estimated using MHC-tetramer (Yee, Savage et al. 1999; Holmberg, Mariathasan et al. 2003; Naeher, Daniels et al. 2007). In our study the relative affinity was analyzed by titration of the peptide, and MHC-tetramers were used as read-out to detect responding peptide-specific $CD8^+$ T cells.

Significant T cell responses were observed to all concentrations of JCV-VP1-p100-peptide down to 10 ng/ml. At the lowest (10 ng/ml) peptide concentration, we measured strong T cell responses, which indicated that these T cells had high-affinity TCRs (Figure 15 B). Similar responses were also observed for CMVpp65-peptide-specific T cells which are known for their high-affinity TCRs (Kiecker, Streitz et al. 2004; Trautmann, Rimbert et al. 2005) (Figure 15 A). Moreover, using the

p100-peptide at concentrations higher than 100 ng/ml, we observed no rise in T cell frequency with serially increased concentrations of the peptide (Figure 15 B). Similar results were observed using the CMVpp65-peptide (Figure 15 A). Using the p36-peptide, the second immunodominant HLA-A2 restricted peptide derived from JCV-VP1, we similarly observed high-affinity T cells at lower (10 ng/ml) concentration. A reasonable explanation for this phenomenon is the fact that T cell responses at this low concentration (10 ng/ml) were already in the plateau of the dose-response curve. Therefore increasing the peptide concentration did not augment the T cell response. It is evident that a low amount of antigen can elicit a T cell response. In other words, high-affinity T cells can bind and respond to a low amount of peptide-HLA complex presented on the APCs (p-HLA density).

Other than density of the p-HLA complex, optimal duration of the interaction of TCR and p-HLA complex (Obst, van Santen et al. 2005; Prlic, Hernandez-Hoyos et al. 2006; Celli, Lemaitre et al. 2007; Celli, Garcia et al. 2008; Henrickson, Mempel et al. 2008) and repeated encounters with the p-HLA complex (Celli, Garcia et al. 2005; Garcia, Pradelli et al. 2007) also have a profound impact on the proliferation and function of T cells. Collectively, this indicates that a low amount of peptides along with HLA class I sets an activation threshold of p-HLA density during the primary infection in HDs which favours the stimulation of high-affinity T cells (Viola and Lanzavecchia 1996). Below and above this activation threshold window, T cells will not get activated or become anergic. In our study, we did not measure the activation window and density of p-HLA on the APCs. However, we observed high T cell responses at 100 ng/ml concentration of p100-peptide. This indicates that

presumably at this concentration of peptide an optimum TCR-p-HLA interaction had occurred.

Measuring the specificities of TCR using MHC-tetramers does not give any information on the functional avidity of T cells which is characterized by inflammatory (cytokine release), antiviral (cytotoxicity) and proliferative responses. In order to characterize the functional reactivity of high affinity T cells, we measured CD137 on the surface of T cells as suggested by other reports (Pollok, Kim et al. 1993; Pollok, Kim et al. 1995; Wehler, Karg et al. 2008). We observed that high-affinity polyomavirus-specific T cells were all functionally active as well (Figure 15 C). In this context, one report showed that high-affinity $CD8^+$ T cells stimulated with a low dose of viral-peptide antigen were functionally more active than those T cells stimulated with high dose (Alexander-Miller, Leggatt et al. 1996). Similarly, in a tumor (melanoma) model, it was reported that a low dose of antigen stimulates high-affinity functional T cells (Bullock, Mullins et al. 2003; Lovgren, Baumgaertner et al. 2012).

Furthermore, to assess the longevity of high-affinity T cells in *in vitro* cultures, we compared p100-specific T cells stimulated with high concentration of the peptide (10 µg/ml) and low concentration of the peptide (100 ng/ml) for four weeks (Figure 16 A, B). We observed that repeated stimulation by low concentrations of the peptide dramatically increased the number of MHC-tetramer positive T cells (Figure 16 B). On the other hand, repeated stimulation by high concentration of the peptide (10 µg/ml) did not have any adverse effect till the third week of stimulation. However after the 4^{th} stimulation almost no peptide-specific T cells were detected (Figure 16 A). These results were in-line with a study, where T cells stimulated with higher

concentrations of peptides in the *in vitro* culture underwent activation induced cell death (AICD) (Schamel, Arechaga et al. 2005).

Novel TCEs derived for JCV-VP1

In the present study, we explored $CD8^+$ T cell responses with the objective to identify new TCEs specific for JCV toward future vaccination approaches. For this purpose, we focused on two alleles, HLA-A2 and HLA-B7 expressed in 50% and in 25% of the Caucasian population, respectively. HLA-A2 and HLA-B7 positive HDs were first screened for the presence of T cell responses to JCV infection. Two routinely practiced methods for the measurement of JCV infection in individuals are anti-VLP antibodies (in the serum) and JCV-DNA in the urine (Jelcic, Aly et al. 2013). However, these two methods do not measure T cell responses. To select HDs based on the T cell reactivity to JCV, we therefore used the VP1-peptide pool as stimulus in a T cell assay (MLPC). VP1 is one out of five proteins of JCV, which has been considered to be highly immunogenic (Jelcic, Aly et al. 2013). Immunogenicity of VP1 was also confirmed in studies with BKV in HDs and patients (Kiecker, Streitz et al. 2004; Hammer, Brestrich et al. 2006; Ginevri, Azzi et al. 2007; Comoli, Hirsch et al. 2008). In this study, considering VP1-protein as the immunological target, we performed epitope mapping using an approach displayed in Figure 24.

JCV-VP1 overlapping peptides and the peptide pool

In a comprehensive epitope mapping strategy, we used JCV-VP1 overlapping peptides as matrix peptide sub-pools in ELISPOT assays (Figure 13). The use of overlapping peptides for T cell epitope mapping for other viruses has already been reported (Trivedi, Williams et al. 2005; Homann, Lewicki et al. 2007; Schalich,

Vytvytska et al. 2008; Wu, Zanker et al. 2011; Walsh, Sidney et al. 2013). In the experimental setting, we used 25,000 to 30,000 effector cells stimulated in MLPC and the IFN-γ secretion was characterized into weak, moderate and strong T cell responses on the basis of S.I. Based on this criteria, we detected JCV-VP1-specific $CD8^+$ T cell responses in almost 73% (16 out of 22) of HDs. Our results of JCV-specific T cell responses were similar to one study where it showed 77.6% positivity using VP1-peptide pool (Jelcic, Aly et al. 2013).

Virus like particles (VLP)

To study cellular immune responses to JCV-VP1 and to map TCEs, we used VP1-VLP as primary stimulus. VLP are derived from VP1 and since VP1 constitutes almost 80% of the JCV-capsid, VLP shows morphological (Teunissen, de Raad et al. 2013) and immunological resemblance to the virus in its natural form (Viscidi, Rollison et al. 2003; Viscidi and Clayman 2006; Nicol, Touze et al. 2012). Some of the previous studies reported the use of polyomavirus-infected cell lysate to study T cell responses (Drake, Moser et al. 2000; Li, Melenhorst et al. 2006). Lysate might constitute all the proteins present in the virus. However, standardization steps (e.g. protein concentration, freeze-thaw etc.) are difficult to be taken and a higher bio-safety (S2) facility is needed to work with polyomaviruses. On the other hand, VLP can be safely used at the S1 level with much less standardization steps. Another big advantage using VLP is the selection of naturally processed T cell specificities. While using peptides, one might find some specificity which is normally not presented by virus-infected cells, VLP on the other hand has to be first internalized and then processed by the proteasome machinery into peptides to bind to the HLA class I

molecule. In this process, all the resulting T cell determinants presented by HLA class I molecules are not artificial but naturally processed.

Naturally processed TCEs

An unexpected result was observed in one donor (Table 4, donor#4196) who was positive for the 15mer peptide OP76 (LINRRTPRVDGQPMY). In contrast, the corresponding 9mer peptide p306 (TPRVDGQPM) predicted to bind with high-affinity was unable to stimulate IFN-γ secretions. One obvious reason might be that the predicted epitope was not the natural TCE (Eisenlohr, Yewdell et al. 1992). The epitope could be a 8mer or 10mer peptide and not a 9mer. In this context, there is evidence that some unusually long peptides derived from viral-antigens (12 to 14 amino acids long) are naturally presented by HLA class I molecules (Schirmbeck, Melber et al. 1994; Longmate, York et al. 2001; Green, Miles et al. 2004; Crough, Burrows et al. 2005; Pudney, Leese et al. 2005; Tynan, Borg et al. 2005; Tynan, Burrows et al. 2005; Burrows, Rossjohn et al. 2006). Moreover, it has been reported that 9mers or 10mers which are completely preserved in immunogenic long peptides, fail to induce T cell responses despite having high predictive HLA class I binding scores. For example, the HLA-A*201 binding p53 nonamer sequence (GLAPPQHLI) has a high predictive score but is non-immunogenic. However, the 11-mer peptide sequence (GLAPPQHLIRV) is comparatively immunogenic (Theobald, Biggs et al. 1997). Similar results were reported for 11mer peptide epitopes derived from the EBV nuclear antigen 1 (Blake, Lee et al. 1997) and the hepatitis B virus (Missale, Redeker et al. 1993). However, to address this question all possible permutations of peptides of different length derived from immunogenic 15mer should be analyzed.

Restriction analysis

Another important aspect in the identification of TCEs was to perform HLA restricted peptide recognition. To this end, HLA-transfectants (K562-A2, K562-B7) and T2 cells were used. The use of K562-A2 (Britten, Meyer et al. 2002) and T2 (Schalich, Vytvytska et al. 2008) has been shown in earlier reports. Employing HLA-transfectants in ELISPOT assays was the key step because that way we were selecting a particular HLA restricted T cell specificity out of many VLP-stimulated HLA class I restricted T cell specificities.

Immune responses to peptides p36 and p100

Previously, two $CD8^+$ TCEs (p36 and p100) have been described in HLA-A2 positive donors (Koralnik, Du Pasquier et al. 2002; Du Pasquier, Kuroda et al. 2003). Using the p100-peptide at different concentrations we characterized T cells with high-affinity TCR (discussed in 3.3). In our experimental setting, the p100-peptide was represented by the 15mer peptide OP25 and matrix sub-pools 5 and 13 (Figure 13). The p36-peptide was covered by the 15mer peptide OP9 and matrix sub-pools 9 and 11. We found $CD8^+$ T cells specific to both immunodominant peptides in more than one-third HLA-A2 positive donors (Table 3). The results of JCV were similar to a study performed in HLA-A2 positive HDs using the immunodominant BKV peptides p44 and p108 (van Aalderen, Remmerswaal et al. 2013).

New TCEs

In addition to two known epitopes, we observed several other HLA-A2 and HLA-B7 restricted T cell specificities (Table 3 and Table 4). Some of them tested positive in more than 10% donors, e.g. the HLA-A2 restricted peptides OP28, OP29, OP72,

OP78, p113 and the HLA-B7 restricted peptides OP23, OP53, p290, and p83. Out of these TCEs, some peptides tested positive in more than 25% of HDs (Table 5). Along with p36 and p100-peptides, these peptides can be also considered as immunodominant. Of most interest are the two neighbouring overlapping 15mer peptides OP28 and OP29 which contain the common 9mer sequence p113 (IGVTSLMNV). Using this 9mer peptide as stimulus we observed high frequencies of positive donors (25%) and strong T cell responses (Table 5).

HLA-immunodominance

Regarding the influence of certain HLA-alleles on cellular immune responses, it has been observed that an individual who is both HLA-A2 and B7 positive, will show a stronger response to the immunogenic epitope restricted to HLA-B7 but not to HLA-A2, a phenomenon called as relative HLA-dominance where HLA-B7 restricted T cell response dominates over the HLA-A2 restricted response. This phenomenon has been explored in CMV (Lacey, Villacres et al. 2003), EBV (Hollsberg 2002) and HIV (Day, Shea et al. 2001). In our study, only one donor#4195 expressed both HLA-A2 and HLA-B7. In this donor, we found that there were moderate to high T cell responses to HLA-A2 restricted epitopes (p100, p36) while only one HLA-B7-binding peptide was found positive. Here, the relative HLA-dominance was not clear. Moreover, it was difficult to explain this phenomenon without the knowledge of any widely accepted JCV-specific HLA-B7 restricted TCE. Nevertheless, the HLA-dominance in JCV is not yet reported and it will be interesting to study this phenomenon, especially in the Caucasian population where one out of every four individuals is both HLA-A2 and HLA-B7 positive.

Cross-reactivity and new TCEs using patients' PBMCs

A patient, who has already experienced an active phase of infection, is the best model to map TCEs, essentially due to the presence of high precursor frequencies of virus-specific T cells. We had the opportunity to use blood samples from an HC-affected patient for TCE mapping. Despite the fact that the patient had experienced BKV-induced HC and not PML (JCV-induced), our hypothesis to identify a TCE was based on the cross-reactivity of T cells due to high sequence homology between these two viruses (Figure 17, 18). For this purpose, we used JCV and BKV-VLP. Interestingly, we observed a similar $CD8^+$ T cell response to both types of VLPs. Additionally, similar responses were observed to a pool of six predicted HLA-B7-binding peptides also (Figure 26). In order to find immunogenic peptides present in the pool, individual peptides were used after being primarily stimulated with JCV-VLP. Two individual peptides (p83 and p306) were found positive (Figure 27). The 9mer peptide p83 was strongly positive in one HD also where it showed high T cell responses in three independent experiments (Table 4).

JCV-specific cellular immunotherapies

To date, single case of T cell adoptive transfer against JCV-infection has been reported (Balduzzi, Lucchini et al. 2011). *In vitro* activated JCV-specific CTLs were introduced in a patient with clinical status of PML who showed great improvement after adoptive T cell transfer. In this case report, T cells were activated using peptide pool of VP1 and LT-antigen with no enrichment and isolation of virus-specific T cells. Bulk T cells, however, might lead to GvHD in immunosuppressed patients due to the presence of non-specific T cells. In our study, we defined some $CD8^+$ TCEs and

since the stimulation was induced by VLP such TCEs are naturally processed. Most importantly, adoptive transfer with viral epitope-specific T cells would greatly minimize the risk of GvHD in patients.

Conclusions

Identification and characterization of polyomavirus-specific T cell epitopes is a prerequisite for the development of both peptide based vaccines and antigen-specific T cell therapies. Adoptive T cell transfer has shown promising clinical results for some virus-related infections (e.g. CMV, EBV, adenovirus etc.). To this end, we exploited a comprehensive strategy including mapping of T cell epitopes using overlapping peptides combined with peptide matrix pools and algorithm programs. It was demonstrated that two immunodominant HLA-A2 restricted T cell specificities for VP1p36 and p100 peptides interact with high avidity and preserve the functional integrity for weeks after *in vitro* culture. Novel, naturally processed T cell epitopes were identified and constitute candidates for future cellular immunotherapies. Our study focused on HLA-A2 and HLA-B7 alleles and as such covers the majority of the Caucasian population. Cross-reactivity, as demonstrated for BKV-and JCV-specific T cells, may also facilitate targeting both polyomaviruses-related diseases simultaneously.

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- Marcel Proust

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SUPPLEMENTARY

S. No.	Peptide Name	Peptide Sequence	S. No.	Peptide Name	Peptide Sequence
1	JCV-VP1-OP1 (15mer)	MAPTKRKGERKDPVQ	2	JCV-VP1-OP2 (15mer)	KRKGERKDPVQVPKL
3	JCV-VP1-OP3 (15mer)	ERKDPVQVPKLLIRG	4	JCV-VP1-OP4 (15mer)	PVQVPKLLIRGGVEV
5	JCV-VP1-OP5 (15mer)	PKLLIRGGVEVLEVK	6	JCV-VP1-OP6 (15mer)	IRGGVEVLEVKTGVD
7	JCV-VP1-OP7 (15mer)	VEVLEVKTGVDSITE	8	JCV-VP1-OP8 (15mer)	EVKTGVDSITEVECF
9	JCV-VP1-OP9 (15mer)	GVDSITEVECFLTPE	10	JCV-VP1-OP10 (15mer)	ITEVECFLTPEMGDP
11	JCV-VP1-OP11 (15mer)	ECFLTPEMGDPDEHL	12	JCV-VP1-OP12 (15mer)	TPEMGDPDEHLRGFS
13	JCV-VP1-OP13 (15mer)	GDPDEHLRGFSKSIS	14	JCV-VP1-OP14 (15mer)	EHLRGFSKSISISDT
15	JCV-VP1-OP15 (15mer)	GFSKSISISDTFESD	16	JCV-VP1-OP16 (15mer)	SISISDTFESDSPNR
17	JCV-VP1-OP17 (15mer)	SDTFESDSPNRDMLP	18	JCV-VP1-OP18 (15mer)	ESDSPNRDMLPCYSV
19	JCV-VP1-OP19 (15mer)	PNRDMLPCYSVARIP	20	JCV-VP1-OP20 (15mer)	MLPCYSVARIPLPNL
21	JCV-VP1-OP21 (15mer)	YSVARIPLPNLNEDL	22	JCV-VP1-OP22 (15mer)	RIPLPNLNEDLTCGN
23	JCV-VP1-OP23 (15mer)	PNLNEDLTCGNILMW	24	JCV-VP1-OP24 (15mer)	EDLTCGNILMWEAVT
25	JCV-VP1-OP25 (15mer)	CGNILMWEAVTLKTE	26	JCV-VP1-OP26 (15mer)	LMWEAVTLKTEVIGV
27	JCV-VP1-OP27 (15mer)	AVTLKTEVIGVTSLM	28	JCV-VP1-OP28 (15mer)	KTEVIGVTSLMNVHS
29	JCV-VP1-OP29 (15mer)	IGVTSLMNVHSNGQA	30	JCV-VP1-OP30 (15mer)	SLMNVHSNGQATHDN
31	JCV-VP1-OP31 (15mer)	VHSNGQATHDNGAGK	32	JCV-VP1-OP32 (15mer)	GQATHDNGAGKPVQG
33	JCV-VP1-OP33 (15mer)	HDNGAGKPVQGTSFH	34	JCV-VP1-OP34 (15mer)	AGKPVQGTSFHFFSV
35	JCV-VP1-OP35 (15mer)	VQGTSFHFFSVGGEA	36	JCV-VP1-OP36 (15mer)	SFHFFSVGGEALELQ
37	JCV-VP1-OP37 (15mer)	FSVGGEALELQGVLF	38	JCV-VP1-OP38 (15mer)	GEALELQGVLFNYRT
39	JCV-VP1-OP39 (15mer)	ELQGVLFNYRTKYPD	40	JCV-VP1-OP40 (15mer)	VLFNYRTKYPDGTIF
41	JCV-VP1-OP41 (15mer)	YRTKYPDGTIFPKNA	42	JCV-VP1-OP42 (15mer)	YPDGTIFPKNATVQS
43	JCV-VP1-OP43 (15mer)	TIFPKNATVQSQVMN	44	JCV-VP1-OP44 (15mer)	KNATVQSQVMNTEHK

S. No.	Peptide Name	Peptide Sequence	S. No.	Peptide Name	Peptide Sequence
45	JCV-VP1-OP45 (15mer)	VQSQVMNTEHKAYLD	46	JCV-VP1-OP46 (15mer)	VMNTEHKAYLDKNKA
47	JCV-VP1-OP47 (15mer)	EHKAYLDKNKAYPVE	48	JCV-VP1-OP48 (15mer)	YLDKNKAYPVECWVP
49	JCV-VP1-OP49 (15mer)	NKAYPVECWVPDPTR	50	JCV-VP1-OP50 (15mer)	PVECWVPDPTRNENT
51	JCV-VP1-OP51 (15mer)	WVPDPTRNENTRYFG	52	JCV-VP1-OP52 (15mer)	PTRNENTRYFGTLTG
53	JCV-VP1-OP53 (15mer)	ENTRYFGTLTGGENV	54	JCV-VP1-OP54 (15mer)	YFGTLTGGENVPPVL
55	JCV-VP1-OP55 (15mer)	LTGGENVPPVLHITN	56	JCV-VP1-OP56 (15mer)	ENVPPVLHITNTATT
57	JCV-VP1-OP57 (15mer)	PVLHITNTATTVLLD	58	JCV-VP1-OP58 (15mer)	ITNTATTVLLDEFGV
59	JCV-VP1-OP59 (15mer)	ATTVLLDEFGVGPLC	60	JCV-VP1-OP60 (15mer)	LLDEFGVGPLCKGDN
61	JCV-VP1-OP61 (15mer)	FGVGPLCKGDNLYLS	62	JCV-VP1-OP62 (15mer)	PLCKGDNLYLSAVDV
63	JCV-VP1-OP63 (15mer)	GDNLYLSAVDVCGMF	64	JCV-VP1-OP64 (15mer)	YLSAVDVCGMFTNRS
65	JCV-VP1-OP65 (15mer)	VDVCGMFTNRSGSQQ	66	JCV-VP1-OP66 (15mer)	GMFTNRSGSQQWRGL
67	JCV-VP1-OP67 (15mer)	NRSGSQQWRGLSRYF	68	JCV-VP1-OP68 (15mer)	SQQWRGLSRYFKVQL
69	JCV-VP1-OP69 (15mer)	RGLSRYFKVQLRKRR	70	JCV-VP1-OP70 (15mer)	RYFKVQLRKRRVKNP
71	JCV-VP1-OP71 (15mer)	VQLRKRRVKNPYPIS	72	JCV-VP1-OP72 (15mer)	KRRVKNPYPISFLLT
73	JCV-VP1-OP73 (15mer)	KNPYPISFLLTDLIN	74	JCV-VP1-OP74 (15mer)	PISFLLTDLINRRTP
75	JCV-VP1-OP75 (15mer)	LLTDLINRRTPRVDG	76	JCV-VP1-OP76 (15mer)	LINRRTPRVDGQPMY
77	JCV-VP1-OP77 (15mer)	RTPRVDGQPMYGMDA	78	JCV-VP1-OP78 (15mer)	VDGQPMYGMDAQVEE
79	JCV-VP1-OP79 (15mer)	PMYGMDAQVEEVRVF	80	JCV-VP1-OP80 (15mer)	MDAQVEEVRVFEGTE
81	JCV-VP1-OP81 (15mer)	VEEVRVFEGTEELPG	82	JCV-VP1-OP82 (15mer)	RVFEGTEELPGDPDM
83	JCV-VP1-OP83 (15mer)	GTEELPGDPDMMRYV	84	JCV-VP1-OP84 (15mer)	LPGDPDMMRYVDKYG
85	JCV-VP1-OP85 (15mer)	PDMMRYVDKYGQLQT	86	JCV-VP1-OP86 (15mer)	MRYVDKYGQLQTKML